RESEARCH PAPER

Pharmacological Assessment of Protein Loaded Nanoparticles for Anticancer Activity

Mallikarjuna Gandla[1], Niranjan Babu Mudduluru[2]*

[1]*Research Scholar, Department of Pharmacology, JNT University Anantapur, Ananthapuramu – 515002, Andhra Pradesh, India.*
*[2]*Professor, Department of Pharmacognosy, Seven Hills College of Pharmacy, Tirupati – 517561, Andhra Pradesh, India.*

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ABSTRACT
The research was focused on assessing the pharmacological efficacy of protein-loaded nanoparticles against dinitrosoethylamine and carbon tetrachloride-induced hepatocellular carcinoma. Four individual groups of forty eight swiss-albino male mice were designed and induced with a single dose of dinitrosethylamine at 200 mg/kg body weight two weeks before administration of a 2 mL/kg dosage of carbon tetrachloride administered once a week along with 5-Flourouracil at 10 mg/kg, b.wt., two times a week for 12 weeks. Plasma, tissue, inflammatory, and cancer-specific indicators were assessed for anti-cancer efficacy of protein-loaded nanoparticles in comparison to 5-fluorouracil (5-FU). Histological analysis was conducted to examine tissue architecture and validate the results of the study. Lactoferrin-loaded solid lipid nanoparticles (LSLN) have demonstrated a significant ameliorative effect in restoring abnormal levels of plasma, tissue, inflammatory, and cancer-specific markers, as evidenced by histopathological examinations compared to group II hepatoma and group III 5-FU. The study has demonstrated that primary dietary proteins designed nanoparticles inhibit tumor development by enhancing the antioxidant system's capacity to reduce cancer cells..
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INTRODUCTION

Making therapeutic proteins at the nanoscale greatly boosts the science and biotechnology behind creating reliable and more effective medications. The area of diagnosis, bioimaging, treatments and infection/cancer prevention has seen amazing results that highlight their potential[1]. Since only around 22–35% of patients survive liver cancer for a decade, considered third most frequent cause of mortality for hepatocarcinoma globally and the fifth most frequently occurring cancer. There are plenty of causes for hepatocellular carcinoma, like metabolic syndrome, drinking alcohol, infections by HCV and HBV viruses, poor genes and foods that contain aflatoxin and N-nitrosamines[2,3]. Chemotherapy is most often used to support and assist with survival in patients, a process called palliative chemotherapy. Nonetheless, taking these drugs for years can bring many negative side effects for the patient, making keeping up with treatment a problem[4,5].

Mammalian exocrine gland epithelial cells make the 80-kDa multifunctional cationic glycoprotein called Lactoferrin (Lf) which is related to the transferrin superfamily. Almost 70% of its amino acids are the same as those found in pigs, mice and cows[6]. Lf is made up of two globular domains, C lobe (345–692) and N lobe (1-333). Also, some forms of helices and folding are called a-helix or b-fold patterns[7] and a metal chelation area that is attached to the protein via four amino acid residues. Thanks to its ability to move iron-ions across a varying pH values and help control iron inside the body, Lf is considered a well-known transferrin. Lf releases its structure when iron binds; apo-Lf is open and flexible, but holo-Lf has a closed pattern. Such changes influence the stability of Lf and certain features of its function. Lf can connect with iron as well as Al^{3+}, Cu^{2+}, Mn^{3+} and Zn^{2+} which all play a part in the way Lf is structured, remains stable and functions[8]. Its ability to bind cationically and its glycosylation allow protein to interact with DNA, LPS and heparin[9]. All of these features help to explain the ways in which Lf fights inflammation, infections, oxidative damage, regulates the immune system, influences beneficial gut bacteria and helps reduce cancer[10-12].

Materials & Methodology

Lactoferrin sample was procured from Sigma-Aldrich. Diethylnitrosamine (DEN), 5-Flourouracil (5-FU) sample and Carbon Tetra chloride (CCl_4) was obtained from Sigma-Aldrich and Merck, India. Chemical reagents used in the study were kept within the defined analytical standards. The reagent kits we used were ordered from Span Diagnostic Private Limited in Mumbai.

**Author for Correspondence: principal.cq@jntua.ac.in*

Formulation of Protein Loaded Solid Lipid Nanoparticle

Optimized Lactoferrin loaded SLN's were successfully developed by a homogenization preceeded by ultra-sonication technique at a suitable temperature range. A hot aqueous phase containing Lactoferrin and Span 80 were added to the lipid phase containing glycerylbehenate (5 %w/w), poloxamer 188 (2 %w/w) and sodium cholate (1.5 %w/w) to obtain water in oil (w/o) microemulsion. Homogenization time was optimized to 3 min to reduce the particle size below 1µm probe sonicator was used. The prepared microemulsion was subjected to sonication for 25 min to get SLN's ranging 30-100nm with narrow size distribution. The LSLN formulation was stored at a room temperature. During lyophilisation mannitol (2%) was added which act as a cryoprotectant[13].

***In-vivo* experimentation procedure**

Animals were kept under biomedical lab conditions and study was performed on swiss albino male mice with an average weight of 25 to 30gms procured from Kedhar Biolabs in Telangana State. The animals were raised in conditions with normal temperature and humidity on a 12hr light/dark cycles. Mice had free access to water and feed at all time during the experimentation period. Animal Ethics Committee of Seven Hills College of Pharmacy, Tirupati certified and authenticated the experiment protocol.

Intoxication of Hepatocellular Carcinoma (HCC) in mice model

DEN was dissolved in physiological normal saline and then induced i.p. at a dose of 200mg/kg b.wt. to every rat except the vehicle control. After the first two weeks, animals were given a subcutaneous injection (s.c) of 2 ml/kg in 1:1 CCl_4 dissolved in olive oil (v/v) weekly for 12 weeks to trigger liver cancer[14].

Grouping of animal & Experimentation

Forty eight albino Swiss mice were assigned to groups: Group I was given water mixed with Tween 80; Group II received targeted doses of DENA/CCl_4 over specified period; Group III was dosed with DENA/CCl_4 and also given 5-FU at 10mg/kg weekly twice for 12 weeks; Group IV was treated with DENA/CCl_4 and taken 60 mg/kg of optimized Lactoferrin loaded solid lipid nanopartices orally for 12 weeks.

One hour after the experiment ended, all mice were anaesthetized with 40mg/kg of thiopental sodium given intraperitoneally and blood was drawn from the heart. The samples were centrifuged at 4°C, 3000rpm for 10min to get the plasma. Using the Microlab 400 Biochemistry analyzer and standard kits, Plasma Serum Glutamate Oxalate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), Alkaline Phosphatase (ALP), Gamma Glutamyl Transaminase (GGT), Lactate Dehydrogenase (LDH), Total Bilirubin (TB) and Total Proteins (TP)[15-20]. IL6 and TNF-α levels were analyzed by ELISA with specific kits. Enzyme Linked Immune Sorbent Assay (ELISA) was used to evaluate Plasma alpha-fetoprotein (AFP), Carcinoembryonic antigen (CEA), vascular Endothelium Growth Factor (VEGF) and Glypican-3 (GPC3) levels[21,22]. Tissue sections from the liver were submersed in formalin and then tested for the presence of Malondialdehyde (MDA), Catalase (CAT) and Super oxide dismutase (SOD) by performing standard-based colorimetric methods[23-25].

Histopathological Studies

Following drying with 100% ethanol, the liver tissues were stored in 10 % buffered formalin and paraffin fixed. Next, the tissues were sectioned into specimen 3 to 5 µm thick using a Weswox rotary microtome, hematoxylin and eosin-stained and visualized under a microscope.

Statistical Evaluation

Values were analyzed by a one way ANOVA with Bonferroni test via GraphPad Prism (v5). A statistically significant of $p<0.05$ was used in the study. Mean values with associated standard error mean were presented.

RESULTS & DISCUSSION

Effect of LSLN on hepatic markers in DEN/CCl_4 administered mice

Values of SGOT, SGPT and ALP were much higher in DEN/CCl_4 administered groups than in the control group ($p<0.05$), based on liver function estimation. As compared to the hepatoma group, 5-FU & LSLN greatly improved each measure of liver function in the DEN/CCl_4 mice depicted in Fig 1 & 2[26-30].

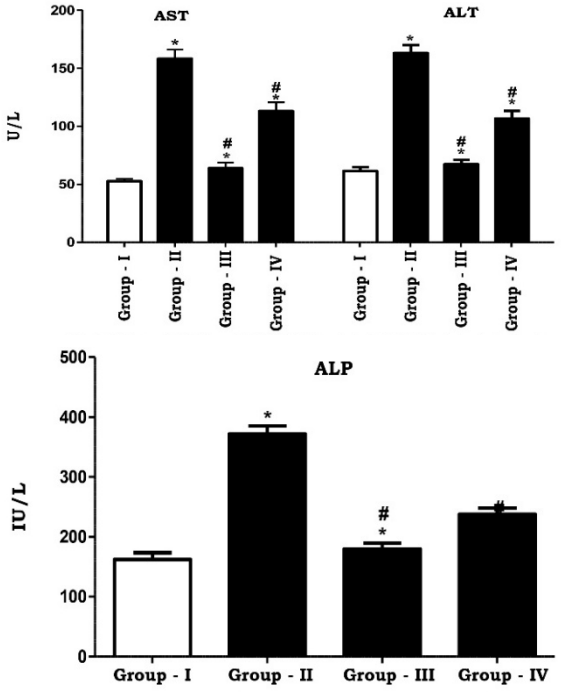
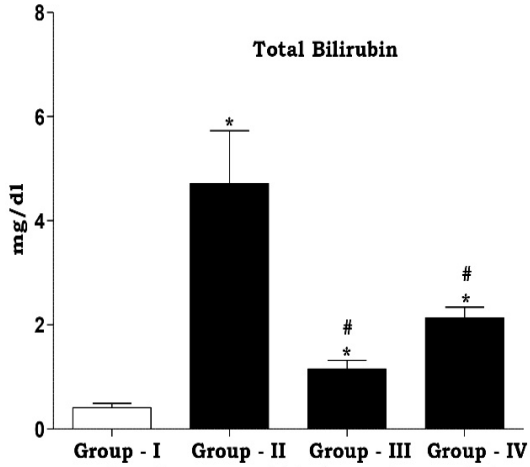


Fig 1: Impact of LSLN on AST & ALT values **Fig 2: Impact of LSLN on ALP values**

Values (n = 12) were displayed using mean±SEM. One-way ANOVA was used to analyze the data, and then Graph Pad Prism version 5 was used to perform the Bonferroni test. The symbol (#) denotes the results are different from group II, while the asterisk (*) indicates that they are statistically different from those in group I.

Impact of LSLN on plasma total bilirubin levels and total-protein in mice received DEN/CCl_4

The total amount of bilirubin was markedly greater while total protein decreased noticeably in DEN/CCl_4 subjected animals compared with group I ($p<0.05$). Within the hepatoma group, 5-FU & LSLN reduced the abnormally high markers presented in Fig 3 & 4[31-35].



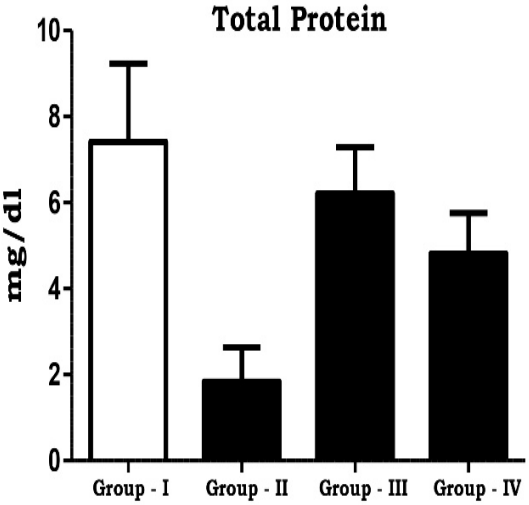
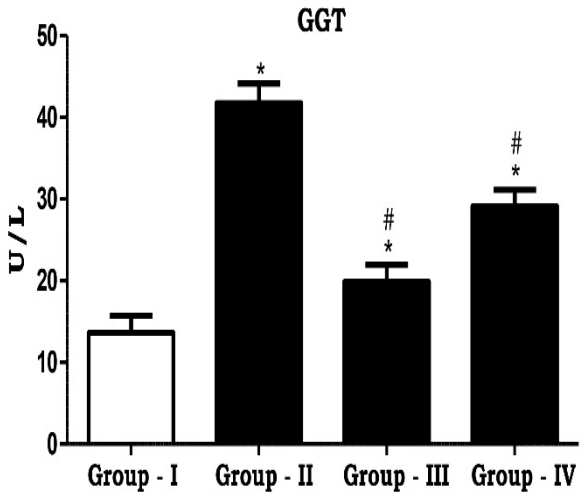


Fig 3: Impact of LSLN on Total Bilirubin **Fig 4: Impact of LSLN on Total Protein**

Values (n = 12) were displayed using mean±SEM. One-way ANOVA was used to analyze the data, and then Graph Pad Prism version 5 was used to perform the Bonferroni test. The symbol (#) denotes the results are different from group II, while the asterisk (*) indicates that they are statistically different from those in group I.

LSLN on GGT and LDH of DEN/CCl_4 intoxicated mice

A rise ($p<0.05$) in GGT & LDH levels was noticed for mice in group–I after being administered DEN/CCl_4. However, after 5-FU & LSLN treatment, both GGT & LDH were greatly ($p < 0.05$) reduced as comparison to group II hepatoma as in Fig 5 & 6[36, 37].



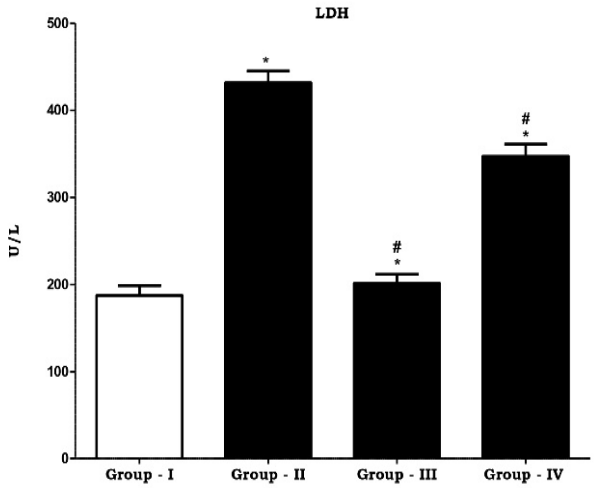
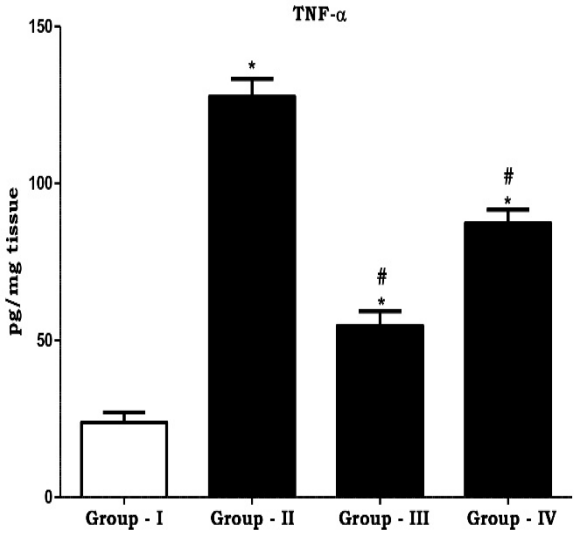


Fig 5: Effect of LSLN on GGT levels **Fig 6: Effect of LSLN on LDH levels**

Values (n = 12) were displayed using mean±SEM. One-way ANOVA was used to analyze the data, and then Graph Pad Prism version 5 was used to perform the Bonferroni test. The symbol (#) denotes the results are different from group II, while the asterisk (*) indicates that they are statistically different from those in group I.

LSLN Role on Levels of Inflammatory markers

A significant ($p<0.05$) increase in IL-6 and TNF-α with DEN/CCl_4 compared to group I was recorded, but the treatments markedly decreased both of these markers ($p<0.05$) in comparison to untreated group II given in Fig 7 & 8.



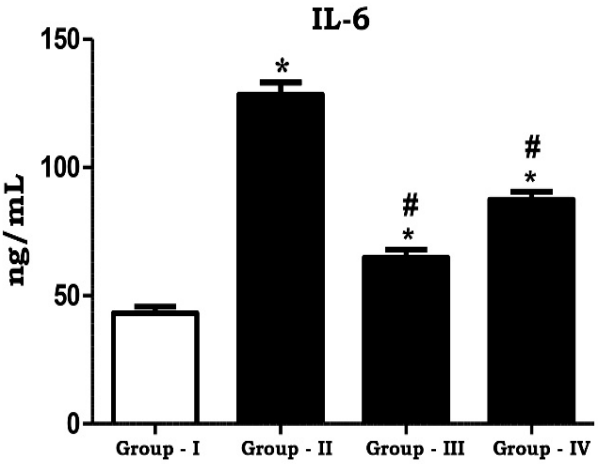
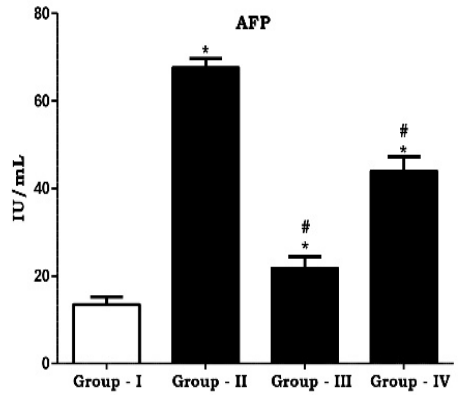
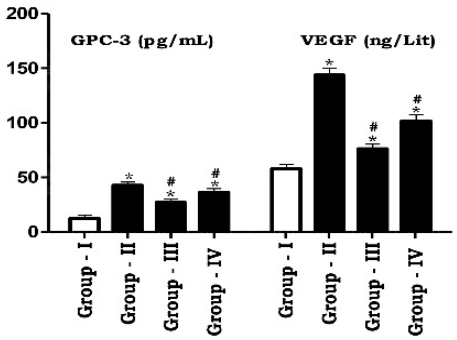


Fig 7: Impact of LSLN on TNF-α levels **Fig 8: Impact of LSLN on IL-6 levels**





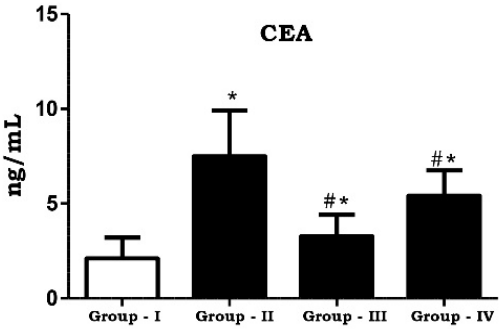


Fig 9: AFP content **Fig 10: GPC-3 & VEGF Levls** **Fig 11: CEA levels**

Values (n = 12) were displayed using mean±SEM. One-way ANOVA was used to analyze the data, and then Graph Pad Prism version 5 was used to perform the Bonferroni test. The symbol (#) denotes the results are different from group II, while the asterisk (*) indicates that they are statistically different from those in group I.

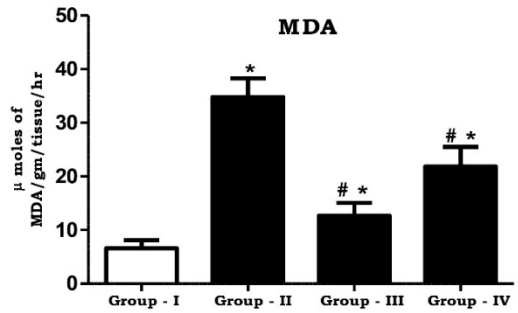
LSLN influenced specific HCC markers found in mice poisoned with DEN and CCl_4

Mice treated with DEN/CCl_4 showed significantly higher ($p<0.05$) levels of AFP, CEA, GPC-3 and VEGF compared to group I, as shown by estimates of the HCC markers. Compared to group II hepatoma, group I with 5-FU & LSLN showed better levels of these markers ($p < 0.05$) as in Fig 9, 10 & 11.

Values were given as mean ± standard error of the mean (n=12). The results were analyzed with one way ANOVA and compared using the Bonferroni method with Graph Pad Prism version 5. A difference marked by * indicates group I is significantly different from the comparison group, while # marks a difference for group II.

The role of LSLN on antioxidant and oxidant markers

MDA in group II was significantly high ($p<0.05$) compared to control group I. Group II had lower CAT and SOD tissue content in comparison to control group I. Both 5-FU and LSLN significantly improved ($p < 0.05$) the tissue pro-oxidant condition and anti-oxidant levels compared to group II given in Fig 12 & 13.



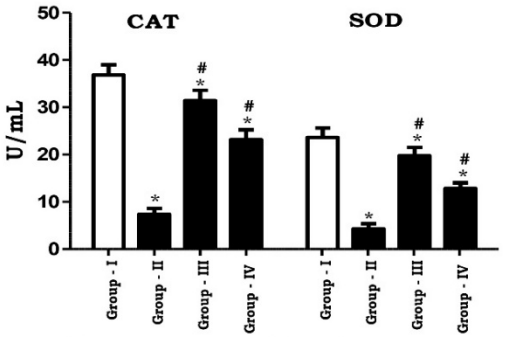


Fig 12: Impact of LSLN on MDA levels **Fig 13: Impact of LSLN on CAT & SOD**

Values were given as mean ± standard error of the mean (n=12). The results were analyzed with one way ANOVA and compared using the Bonferroni method with Graph Pad Prism version 5. A difference marked by * indicates group I is significantly different from the comparison group, while # marks a difference for group II.

Impact of LSLN on hepatocyte architecture

Hepatocytes in group I remained healthy; sections from group II had ballooning degeneration, here cells contained large eosinophilic inclusions and there were many inflammatory cells with pyknotic nuclei; and 5-FU showed nearly normal architecture; LSLN sections had less pathology and less inflammation with fewer pyknotic nuclei (Fig 14)[38,39].

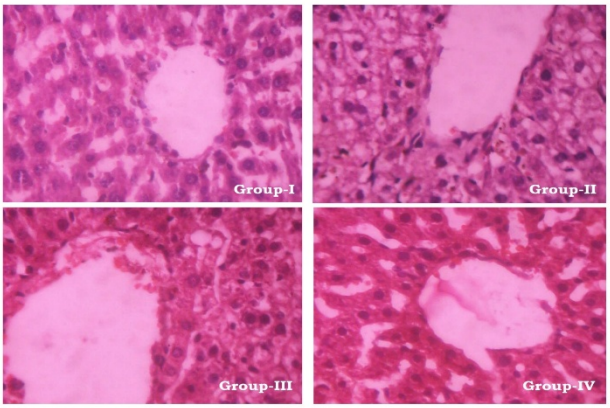


Fig 14: Effect of LSLN on hepatocyte architecture

Discussion

Hepatocellular carcinoma occurs primarily when hepatocytes are changed to cancer because of complex genetic alterations. DEN frequently induces cancer in the liver in the animals it affects. Researchers report that DEN creates ROS and these ROS act on the liver to produce DNA adducts that cause oxidative stress and cell injury. On top of its synthesis inside the body, people can also be exposed to this genetically toxic compound in cigarette smoke, different components found in drugs and in the water around us if it has high nitrate levels[40,41]. Getting proteins in your routine may help your body by strengthening the immunity, resisting dreadful bacteria, virus and yeast keeping your digestive system in good health. It's fascinating that the proteins in milk may suppress tumor growth in different kinds of cancers[42,43]. Hepatocellular carcinoma is frequently developed in experimental rats and mice when DEN is given as an oncogen and genotoxin. Adducts from DNA alkylation by DEN might initiate the development of liver cancer. Hepatocellular carcinoma can be brought about by a group injection of DEN and the cancer promoting agent CCl_4 into a host. Hepatocyte damage carried out by free radicals leads to cell death after exposure to CCl_4. After CCl_4 contacts the hepatocytes, the cells quickly die but some of this death can be restored. In addition, lipid peroxidation results in additional damages to cells[44].

If liver enzyme levels go up, it means that some fluid has leaked out and the cell membrane isn't working properly. Serum SGOT, SGPT and ALP showed a marked increase when DEN/CCl_4 was given. Scientists hypothesize that a rise in these chemicals is linked with injured hepatocytes[45]. Many researchers take γ-GT to be the earliest sign of the development of hepatocarcinogenesis. Elevated γ-GT in the study suggests that DEN/CCl_4 causes hepatocellular damage and parenchymal and necrosis cells in the liver[46].

The rise in LDH expression has been considered to support both glycolysis and decreased use of oxygen by cancer cells[47]. Studies with DEN/CCl_4 induced models revealed higher values of total-bilirubin, suggesting that the liver's normal responses may be blocked and that bilirubin being released unchanged by injured liver cells[48]. The loss of protein is thought to be caused by harm that starts and sits in the endoplasmic reticulum of liver cells. With this damage, P450 can't function normally which leads to a loss in protein synthesis along with the buildup of triglycerides and fat within the liver. The 5-FU & LSLN-treated groups showed higher protein levels which meant their endoplasmic reticulum was regulated for cellular protein synthesis[49]. IL-6 boosts inflammation in the body and promotes the reproduction of liver cancer cells by activating JAK-STAT3 and boosting HCC's chances of becoming more invasive[50]. TNF-α is an important cause of liver inflammation during oxidative stress, causing fibrosis and cell death in the liver. Having been stimulated by xenobiotics, Kupffer cells release the cytokines TNF-α and IL-6 which lead to both hepatic inflammation and damage. TNF-α stimulates events that result in liver toxicity and the development of fibrinogen which then damage the liver because of inflammation[51].

Experts have found that very high levels of AFP are linked to the growth of Hepatocellular carcinoma such as when there is vascular intrusion and satellitosis[52]. Better accuracy for liver tumor detection was found when the tumor markers

AFP and CEA were high, as these enzymes leak out of damaged liver cells into the blood[53]. The GPC-3 protein, part of the glypican group, is made in the fetal liver but only in trace amounts by normal adult hepatocytes. Glypicans help cells grow, change form and move across tissues by interfering with growth factors[54,55]. Research has found that higher circulating VEGF is related to both the growth and development of hepatocellular carcinoma through angiogenesis[56].

Rats that received treatment with DEN/CCl_4 had evidence of elevated reactive oxygen species and peroxidation of lipids in the hepatocytes. So, the products formed from the free radical reaction with the hepatic microsome lipids are related to a reorganization of double bonds into conjugated diene lipids[57]. In animals given DEN, increased oxidative stress and the presence of free radicals are responsible for liver injury, seen as hepatic MDA levels rising and GST, SOD and CAT levels falling after hepatocellular carcinoma induction[26,58].

Examinations of liver samples from groups treated with DEN/CCl_4 revealed greater collagen production, increased infiltration of inflammatory cells, more severe types of steatosis, cell death, and damage to most parts of the liver lobule and ballooning degeneration than in non-treated group I serves. There was a noticeable improvement in hepatic architecture on tissue sections from animals given 5-FU. Although some LSLN treated areas saw an ameliorative effect, hepatocytes across most of the sample displayed a good decrease in microvesicular and macrovesicular fatty liver changes. Even so, dilation of the central vein has allowed inflammatory cells into the nearby tissue and some collagen fibers are still noticeable.

CONCLUSION

The presence of genotoxic chemicals such as DEN often leads to hepatocellular carcinoma (HCC) which forms by complex cellular and molecular mechanisms. Because of DEN, if CCl_4 is used as a promoter, the oxidative stress, inflammation and damage to cells induce hepatocyte transformation and tumors. Higher liver enzyme levels, disturbed architecture and signs from biomarkers AFP and GPC-3 suggest liver cancer. The necrosis and fibrosis in collagen disorders are often caused by elevated TNF-α and IL-6 values. During liver damage, proteins found in biological sources could decrease liver damage and restore part of its function. It was shown in the study that antitumor activity of LSLN against DEN/CCL_4 induced hepatocarcinogenesis was clearly present.

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