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Modulatory Effect of B- Glucans Aginst Nitrosodiethylamine-Induced Hepatocarcinogenesis in Rats by Suppressing NF-Kb/IL-6 Signaling.

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ABSTRACT

β-glucan is one of the most abundant forms of polysaccharides known as biological response modifiers. Accordingly, the aim of the present study is to divulge the efficiency of GLU against N-nitrosodiethylamine induced hepatocarcinogenesis in rats. The results revealed that DEN induced liver damage as evidence by significantly increases in serum indices of liver function enzymes, increase serum levels of tumor markers; alpha fetoprotein and arginase, pro-inflammatory markers; interleukin-6, and hepatic levels of nitric oxide along with transcript of nuclear factor-kappa beta gene, as well as induced significantly high content of hepatic lipid peroxide, and low hepatic content of antioxidant enzymes when compared with the normal control group. However, treatment with preventive and therapeutic GLU, displayed improvement in treated parameters. Histological investigations revealed that DEN treatment affect the hepatic architecture throughout the significant severe appearance of inflammatory cells infiltration in the portal area and congestion in the portal vein in association with severe degeneration and dysplasia in hepatocytes all over hepatic paranchema. Severity of hepatic architecture changes was significantly decreased with both GLU therapeutic and preventive treatments. Given these promising findings, the present study suggests that GLU has the ability to protect against DEN-induced hepatocarcinogensis.

Keywords: Hepatocarcinogensis, β-glucan, Tumor markers**,** NF-κB gene expression, antioxidant enzymes.

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INTRODUTION

The medicinal health benefits and pharmacological potential of both edible and non-edible mushrooms have long been recognized, including their antitumor, immunostimulatory and anti-inflammatory activities. Because their constituents are believed to promote health and longevity, and also due to their taste, texture, high fiber, and low fat contents [1], mushrooms have long been consumed. The biological importance of many mushrooms is primarily related to the great structural diversity of their polysaccharides. The mechanisms involved in the antitumor activity of polysaccharides are not fully known. Most mushroom polysaccharides seem to exert their antitumor activity *via* activation of multiple pathways in the host immune response, or by acting as biological response modifiers [2]. Promising antitumor activity has been attributed to several polysaccharides [3], and many of them are also used as adjuvants in chemotherapy treatment for different types of tumors. Polysaccharides with antitumor action possess a specific chemical composition and molecular configuration and may be in the form of homo- or heteropolymers. Among the molecules with antitumor activity, most belong to the β-d-glucan group [4]. Their structure consists mainly of a β-(1→3)-linked main-chain with branches at O-6, which are believed to be necessary for their antitumor action [5].

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer mortality worldwide [6]. Its occurrence has been increasing steadily over the past decades [7]. The majority of HCC cases are attributable to underlying infections caused by hepatitis B and C viruses [8]. However, several other risk factors, including obesity, iron overload, environmental pollutants, alcohol consumption, as well as several dietary carcinogens, such as aflatoxins and nitrosamines, have been shown to be involved in its etiology [9].

Diethylnitrosamine (DEN) is a well known hepatocarcinogenic agent present in tobacco smoke, ground water, cured and fried meals, cheddar cheese, alcoholic beverages, agriculture chemicals and pharmaceutical products [10]. DEN is an N-nitroso alkyl compound depicts as an effective hepatotoxin to experimental animals, producing toxicity after repeated administration. DEN become metabolically active by the action of cytochrome p450 enzymes to produce reactive electrophiles, which increase oxidative stress level leading to cytotoxicity, mutagenecity and carcinogenicity. Oxidative stress is considered as critical mechanism contributing to DEN induced hepatotoxicity [11]. It has been suggested that increased reactive oxygen species (ROS) might contribute to tumorigenesis by activating NF-κB/IL-6 signal pathway [12]. However, it was still unclear whether the protective effects of β-glucan (GLU) against DEN-induced hepatocarcinogenesis was associated with the suppression of NF-kB pathway, although a previous study has detected ROS overexpression in DEN -induced hepatocarcinogenesis [13]. The current study was designed to evaluate the hepatoprotective effects of GLU against DEN -induced hepatocarcinogenesis and to explore the association between GLU and suppression of NF-kB/IL-6 signaling.

MATERIALS AND METHODS

Glucan Preparation

Glucan was extracted from dry mushroom, *Agaricus bisporus,* obtained from Ploshia Mushroom Company (Egypt). Dry mushroom was added to 0.1mol/L of NaOH and stirred for 30 min at 60°C. The material was then heated to 115°C at 8.5 psi for 45 min and then allowed to settle for 72 hrs. The sediment was resuspended and washed in distilled water by centrifugation at 350 g for 20 minutes. The alkali insoluble solids were combined with 0.1mol/L acetic acid and heated to 85°C for 1 hr, then allowed to settle at 38°C. The acid insoluble solids were drawn off and centrifuged. The compacted solid material was mixed with 3% H_2O_2 and refrigerated for 3 hrs with periodic mixing. The material was then centrifuged and the pellet washed twice with distilled water followed by two washes in 100% acetone. The harvested solid material (glucan powder) was dispersed on drying trays and dried under vacuum at 38°C for 2 hrs in the presence of Ca_2SO_4, and then further dried overnight under vacuum at room temperature [14, 15].

Animals

Thirty adult male Swiss albino rats weighing 200±20g obtained from the National Research Center (Dokki, Giza, Egypt), were housed in standard plastic cages. Rats were kept in the laboratory under controlled conditions of temperature (27±2^{0}C) and humidity (60±5%) with 12h light/12h dark cycles in well ventilated

cages with free access to standard laboratory pellet chow and water *ad libitum*. All the experimental procedures were carried out according to the principles and guidelines of the Ethics Committee of the National Research Centre conformed to "Guide for the care and use of Laboratory Animals" for the use and welfare of experimental animals, published by the US National Institutes of Health (NIH publication No. 85–23, 1996).

Experimental design

After an acclimatization period of 7 days, rats were randomly allocated and divided into five groups of 6 animals each.

- Groups I rats kept as control animals.
- Group II rats were experimentally induced by oral administration of DEN (20mg /kg b.wt. daily for six weeks [16].
- Group III rats were orally administrated daily with GLU for six weeks. (65 mg /kg b.wt. [15].
- Group IV rats were administrated GLU daily as in group III for two weeks then experimentally induced with DEN concomitant with GLU for six weeks (preventive treatment).
- Group V rats were experimentally induced daily with DEN as in group II then administrated with GLU for six weeks (therapeutic treatment).

By the end of 6 weeks, all animals were sacrificed 24 hours after the last treatment under urethane anaesthesia. Blood samples were collected and serum were separated and stored at -20^{0}C pending analyses. A midline abdominal incision was performed in each animal and livers were harvested, perfused with cold isotonic saline and dried carefully.

Determination of Alanine Transferase, Gamma Glutamayl Transferase Activities and Creatinine And Nitric Oxide Levels

Alanine transferase (ALT), gamma glutamayl transferase (GGT) activities and creatinine level were determined in serum using kits purchased from Biometra Co. (Egypt). Arginase activity was determined in serum using commercial kit from Bio-vision incorporated, USA. Nitric oxide (NO) was determined in liver tissue homogenate using kits purchased from Bio diagnostic Co. (Egypt).

Determination of Serum Alpha-Fetoprotein and Interleukin 6 Levels

Levels of alpha-fetoprotein (AFP), interleukin-6 (IL-6) and activity of caspase-3 were determined by using the corresponding ELISA kit following protocols provided by the manufacturers (R&D Systems Kit, USA and DRG, USA).

Determination of Antioxidants and Oxidative Stress Parameters in Hepatic Tissue

The liver was weighed and homogenized (10%) in chilled 50 mmol phosphate buffered saline (pH 7.4), centrifuged at 1200 g, at 4 °C for 15 min, using universal centrifuge (16R, Germany), then the supernatants were used for the determination of the following parameters: Superoxide dismutase (SOD) activity was determined according to **Nishikimi et al. [16],** GSH concentration was measured according to **Beutler et al. [17]**, Lipid peroxide (LPO), in terms of malondialdehyde (MDA) were measured according to the method of **Satoh [18]**.

Detection of Nuclear Factor Kappa B Relative Gene Expression in Liver Tissues by Quantitative Real Time PCR (qRT-PCR)

RNA Isolation and Reverse Transcription

To investigate the changes in mRNA expression for nuclear factor kappa B (NF-kb), total RNA was isolated from 100mg liver using TRIzol reagent (Life Technologies, USA) in accordance to the manufacturer's instructions. RNA integrity was confirmed by 1% agarose gel electrophoresis and stained with ethidium bromide. First strand ccomplementary DNA (cDNA) synthesis was performed with reverse transcriptase (Invitrogen) according to the manufacturer's protocol using 1µg of total RNA as the template.

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Quantitative Real-Time Polymerase Chain Reaction (qPCR)

RT-PCRs were performed in a thermal cycler step one plus **(Applied Biosystems, USA)** using Sequence Detection Software **(PE Biosystems, CA).** A reaction mixture of total volume 25µl consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer and 2µL of cDNA. The sequences of PCR primer pairs used for each gene are as follows, NF-kb: Forward: F: 5'-GCTTACGGTGGGATTGCATT-3', Reverse: R:5'-TTATGGTGCCATGGGTGATG-3', β-actin: Forward: 5'-ATG GGA GTT GCT GTT GAA GTC A-3', Reverse: 5'-CCG AGG GCC CAC TAA AGG-3'. The PCR thermal-cycling conditions included an initial step at 95°C for 5 min; 40 cycles at 95°C for 20s, 60°C for 30s, and 72°C for 20s. Curve analysis was performed at the end of the reaction. The data were normalized using the GAPDH gene that was amplified in each set of PCR experiments. Relative expression of target mRNA was calculated using the comparative Ct method using the **Pfaffl method [19].** Each experiment was performed in triplicate in two independent experiments.

Histopathological Assessment

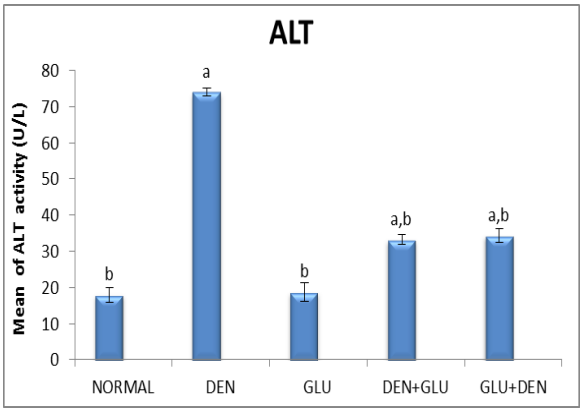
Liver samples were fixed in 10% formal saline for 24 hours. Washing was done in tape water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin and eosin (H&E) stain for routine examination through the light electric microscope [20].

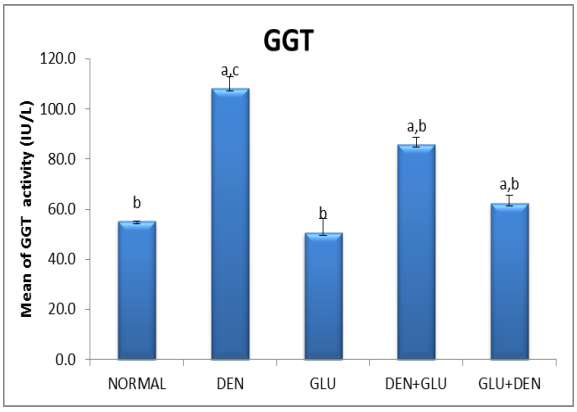
Statistical Analyses

All data were expressed as the mean ± SE. One-way analysis of variance (ANOVA) with Least Significant Difference (LSD) was used to test for differences in means of variables between groups. A probability of $P<0.05$ was considered significant. All data were analyzed by Statistical Package for Social Science (SPSS) version 13.0 for Windows (SPSS® Chicago, IL, USA) software program.

RESULTS

Administration of DEN induced hepatic injury throughout the significant increase in plasma ALT and GGT activities along with AFP and ARG levels in comparison with both controls and GLU treated groups. Preventive and therapeutic administration of GLU showed statistically significant improvement versus the DEN group throughout the exhibited significant modulation in liver enzymes (ALT & GGT) and tumor markers (AFP & ARG) that were augmented in hepatic toxicity (Fig.1).





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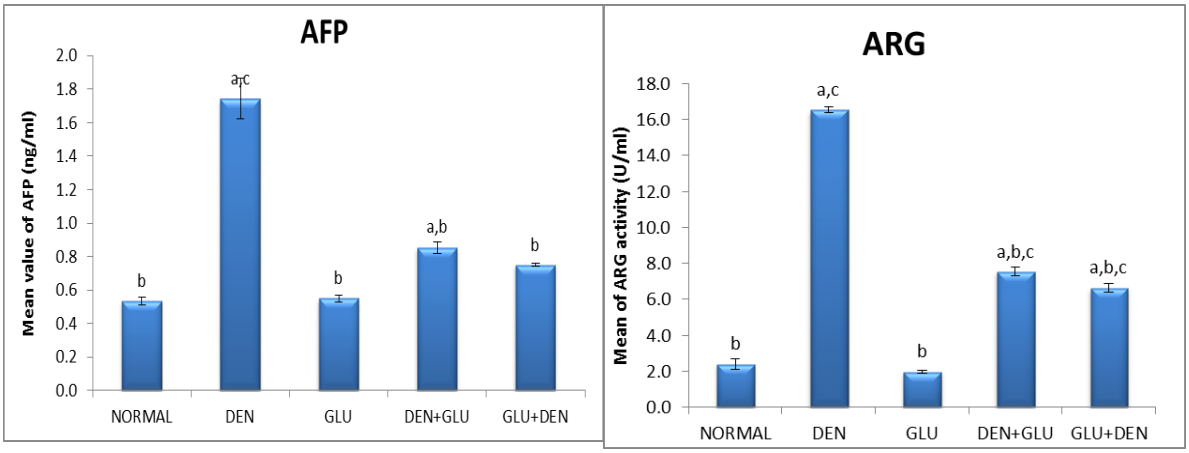

Fig. 1: ALT, GGT, AFP and ARG levels in different treatments compared with controls. Each column represent mean±SE (n=6). [a] P<0.05 compared to control; [b] P<0.05 compared to DEN group.

Additionally, as shown in Fig. 2, the pro-inflammatory markers: NO and IL-6 levels and gene expression of NF-kb were increased in DEN intoxicated rats compared with the control animals. The administration of GLU pre or post DEN treatment decreases the levels of these pro-inflammatory markers in DEN intoxicated rats.

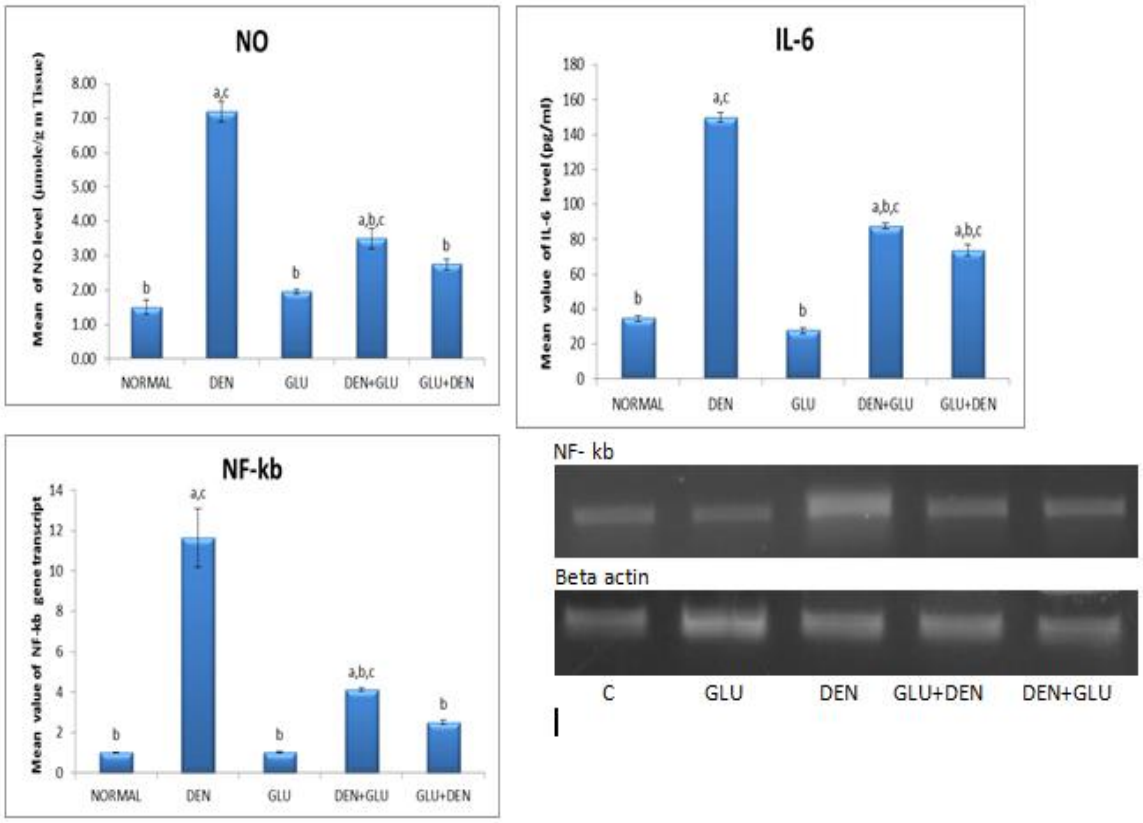

Fig. 2: NO and IL-6 levels and NF-κb gene transcripts level in different treatments compared with controls. Each column represent mean±SE (n=6). a P<0.05 compared to control; b P<0.05 compared to DEN group.

The effect of DEN on endogenous antioxidant status is shown in Fig. 3. DEN induced a significant decrease in the liver SOD activity and GSH content accompanied with a significant increase in MDA levels as compared to the untreated control group. However, pre or post treatment of DEN intoxicated rats; with GLU resulted in a significant amelioration in these parameters.

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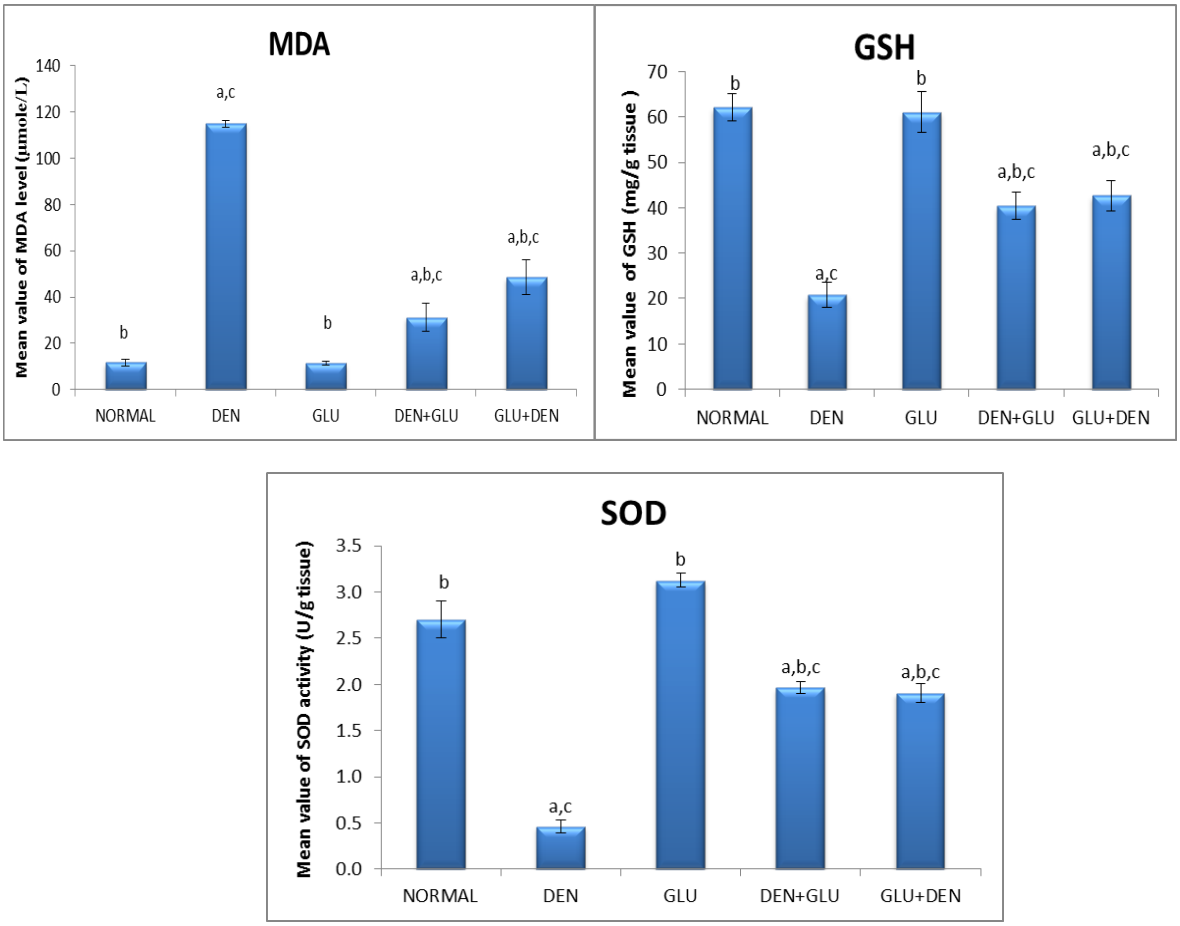
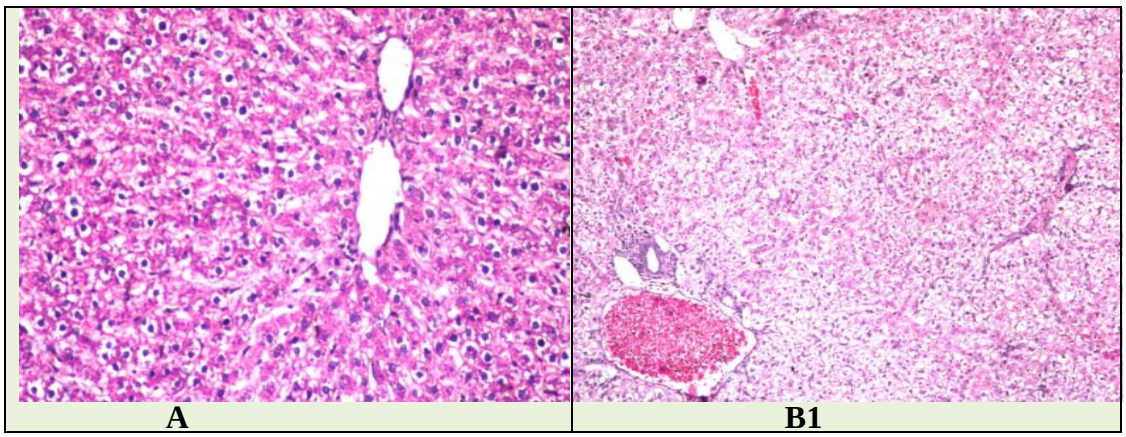


Fig. 3: MDA, GSH and SOD levels in different treatments compared with controls. Each column represent mean±SE (n=6). [a] P<0.05 compared to control; [b] P<0.05 compared to DEN group.

Histopathology

Liver tissues either for control or GLU rat groups showed normal hepatic architecture of normal parenchyma and normal vascular as well as normal stroma with no inflammatory infiltrate or fibrosis in the portal tracts and no degenerative changes (Fig. 5A&C). Liver tissues with DEN treatment showed significant marked severe fibrosis in-between the degenerated dysplastic hepatocytes as well as inflammatory cells infiltration in portal area (Fig. 5B 1&2). With GLU preventive and therapeutic treatment, liver tissues showed fibrosis in the portal area as well as in between the degenerated and dysplastic hepatocytes also portal area showed inflammatory cells infiltration (Fig. 5D & E).



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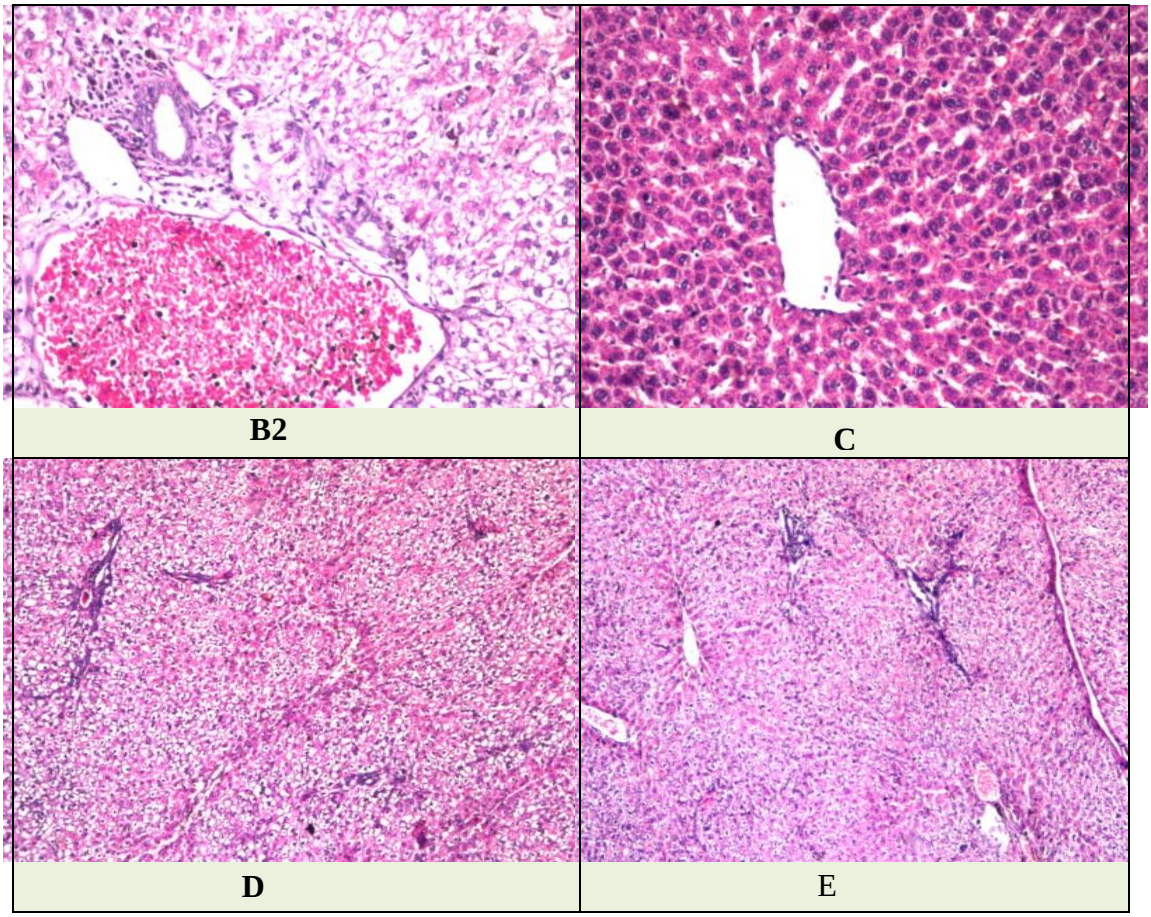

Fig. 5: Photomicrographs of liver specimens stained with haemotoxylin and eosin showing

(A) Normal hepatic architecture in *control* animals.
(B1) The portal area with inflammatory cells infiltration and congestion in portal vein in *DEN* treated rats.
(B2) Fibrosis in-between the degenerated dysplastic hepatocytes in *DEN* treated rats.
(C) Normal hepatic architecture in *GLU* treatment.
(D1) Fibrosis in portal area as well as in-between the degenerated dysplastic hepatocytes in *preventive treatment*
(E1) Fibrosis in portal area and in-between the degenerated dysplastic hepatocytes in therapeutic *treatment*

DISCUSSION

An understanding of how cancer may be prevented is one of the key objectives of the recent researches. This can be achieved to some extent by using chemopreventive agents, naturally occurring or synthetic, that can suppress or prevent the process of tumor development. Therefore, it is essential to identify agents as well as to evaluate their efficacy and to elucidate their mechanisms of action. Many anticancer drugs cause severe adverse effects, including damage to the immune system, which constrains their use in treatment [21]. Thus, it is important to investigate novel antitumor drugs that offer improved immune stimulatory and toxicity profiles. In this regard, many polysaccharides and polysaccharide–protein complexes isolated from mushrooms, fungi, yeasts, algae, lichens, and plants, are attracting attention owing to their immunomodulatory and anticancer effects. β-Glucans, which is found in some foods, are accepted to be one of the most powerful immune response modifiers, especially the low molecular weight β-glucan for the high solubility and low viscosity [22, 23].

It is known that the ALT and GGT serum activities are indicative for hepatic function; their increase is correlated with the hepatic injury [24]. DEN hepatic injury is related to the disturbance in hepatocytes membrane instability and metabolism resulting in alterations of the serum levels of these enzymes. In the present investigation, our data showed that stimulation of liver with DEN leads to tissue damage as was established by the elevated levels of ALT, GGT. The decrease of ALT and GGT serum activities in GLU treated rats may be attributed to the decrease of cellular damage.

Tumor markers like AFP & ARG are potential screening tools that are widely used for early diagnosis of tumors. AFP, the classical gold standard and most commonly used biomarker for HCC, has been recognized in the presence of acute and chronic viral hepatitis as well as in patients with cirrhosis caused by hepatitis C [25]. ARG is expressed predominantly in the liver, and to lesser degrees in the breast, kidney, testes, salivary glands, epidermis and erythrocytes. Its activity is a key diagnostic indicator so it has been reported that, some of the urea cycle enzymes leak rapidly from hepatocytes when liver cells are damaged [26]. Our data indicated a significant increase in serum levels of AFP & ARG of DEN-treated rats. Upon treatment with GLU, the serum levels of the tumor markers; AFP& ARG were significantly declined.

The mechanisms of GLU antitumor activity are not yet understood, and the antitumor activity of other polysaccharides, such as those from mushrooms, can be due to a combination of effects. Another important aspect is the possible selective effect of GLU. However, previous study was done on HepG2 cells, GLU showed a cytotoxic effect without affecting the viability of hepatocytes. This cytotoxic action may be related to their linear structure. This interaction may be more effective in tumor cells than normal cells. Compounds that have toxic effects on tumors and not on normal cells are very promising and promote an increased interest in the application of GLU in cancer therapy [27].

DEN stimulation causes a release of pro-inflammatory markers; IL-6 and NO levels and gene expression of NF-κβ which are abundantly produced by hepatocytes in response to DEN administration that exhibited the extent of liver toxicity. IL-6 is an immunoregulatory cytokines that produced by cancer cells and associated macrophages, its high serum level is associated with specific immune and metabolic alterations that lead to cancer cachexia, one of the main causes of death in cancer patients. IL-6 involved in cancer cells growth through induction of matrix metalloproteinase productions [28], also induction of tumor angiogenesis [29]. Additionally, a novel role of IL-6 signaling identified in assisting NF-κB signaling to synergistically induce the transcription of proinflammatory genes. It was named the “inflammation amplifier [30].

In the same way, NF-κβ is a family of five closely related proteins which are found in several dimeric combinations and bind to the κβ sites on DNA [31]. It is activated by free radicals, inflammatory stimuli, cytokines, carcinogens, tumor promoters or endotoxins. The NF- κβ is translocated to the nucleus and induces cellular transformation, proliferation, invasion, metastasis, and/or inflammation [32]. NF-κβ regulates the genes that control cell proliferation and cell survival. Many different types of human tumors have misregulated NF- κβ; that is, NF- κβ is constitutively active. Active NF- κβ turns on the expression of genes that keeps the cell proliferating and protects the cell from conditions that would otherwise cause it to die *via* apoptosis. In inflammatory cells, continuous NF- κβ activity could promote the production of reactive oxygen species, thereby damaging DNA of surrounding epithelial cells [33].

Inhibition of NF-kB activation in hepatocytes of Mdr22/2 mice retarded and reduced HCC development. The major mechanism by which NF-kB was suggested to exert its tumor promoting function inMdr22/2 mice is the suppression of apoptosis [34]. However, the published results are also consistent with a role for hepatocyte NF-kB in the maintenance of chronic inflammation in Mdr22/2 mice that is critical for tumor development [35].

Aberrant activation of NF- κβ is frequently observed in many cancers. Moreover, suppression of NF-κβ/IL-6 limits the proliferation of cancer cells. In addition, NF- κβ/IL-6 is a key player in the inflammatory response. Hence, the method of inhibiting NF- κβ/IL-6 signaling has potential therapeutic application in cancer and inflammatory diseases. Many natural products involved in anti-cancer and anti-inflammatory activity have been shown to inhibit NF- κβ/IL-6 signaling [33].

Nitric oxide plays an important role in HCC development and its progression [36]. Hepatocytes produce NO in response to several inflammatory stimuli. Tumor cells themselves are able to produce large amounts of NO due to induced expression of inducible nitric oxide synthase (iNOS), which may prevail in rapidly growing tumors. Thus, NO production by hepatic tissue is accelerated in patients with HCC. Increased NO generation is well-recognized as an essential step initiating neoplastic transformation along with HCC development and its progression (metastasis). NO production was significantly increased in liver in the presence of DEN. Since iNOS expression paralleled this increase in NO production it is likely that this is due to iNOS induction [37, 38]. In this study GLU significantly suppressed the NF- κβ gene expression associated with suppression in IL-6 and NO levels, this explain the anti-inflammatory activity of GLU.

Oxidative stress is associated with damage to a wide range of macromolecular species including lipids, proteins, and nucleic acids thereby producing major interrelated derangements of cellular metabolism including peroxidation of lipids. Increased level of MDA was reported during DEN-induced hepatocarcinogenesis. This dynamic action may further lead to uncompromised production of free radicals overwhelming the cellular antioxidant defense [39]. It has been extensively reported that free radicals participated in DEN-induced hepatocarcinogenesis. MDA generation at the initiation stage can be prevented by free radicals scavengers and antioxidant action of GLU. Animals treated with GLU (both pre and post) exhibited significantly lowered the levels of MDA, both in liver and serum, when compared with animals induced with DEN. This shows the antilipidperoxidative role of GLU that is probably mediated by its ability to scavenge free radical generation [40].

Antioxidants possess a variety of biological activities, including the induction of drug-metabolizing enzymes, inhibition of prostaglandin synthesis, inhibition of carcinogen- induced mutagenesis, and scavenging of free radicals [41]. Decreases in the activity of SOD, and GSH level are seen in tumor cells. The compounds that can scavenge excessive free radicals in the body are suggested to hinder the process of carcinogenesis [42]. Such studies support our findings as we had seen a significant decrease in the activities of antioxidant enzyme in liver of animals treated with DEN in comparison with normal animals. In GLU (both preventive and therapeutic group) treated animals, there was a significantly higher level of GSH and SOD activity in liver when compared to DEN-induced animals. The increases in the levels of antioxidant enzymes are consistent with the idea of DNA–carcinogen interaction attenuation and thereby averting a favorable environment for carcinogenesis.

In the present investigation, the histopathological study showed that liver tissues with DEN treatment showed significant severe fibrosis in-between the degenerated dysplastic hepatocytes as well as inflammatory cells infiltration in portal area. With GLU preventive and therapeutic treatments, liver tissues showed significant improvement with β-glucan supplementation upon hepatocytes. These results are in line with those of [43] who observed areas of intracellular vacuolization; sinusoidal dilatation, congestion, and focal necrosis of the liver parenchyma within the ischemia/reperfusion liver injury rat group while these changes were not observed with β-glucan supplementation group suggesting that β-glucan reduced the extent of histological changes. Both pre and post treatment with β-glucan ameliorated liver injury that was attributed to its antioxidant efficacy.

CONCLUSION

In conclusion, the current findings showed that GLU altered serum parameters of hepatic damage induced by DEN and modulated NO and IL-6 levels associated with NF-κβ suppression which contributed to experimental hepatocarcinogenesis in rats. GLU is considered a promising preventive and therapeutic treatment towards hepatocarcinogenesis. Further studies are underway to elucidate the molecular mechanisms involved to prove GLU efficacy as an anticancer agent.

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