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Irradiated β - Glucan attenuates diethyl nitrosamine induced hepatocarcino-genesis through regulation of CD4,CD8 and β – Catenin

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A B S T R A C T

β-glucans are heterogeneous group of natural polysaccharides mostly investigated for their immunological effects. The objective of this study was to evaluate the efficiency of irradiated β-glucan on N-nitrosodiethylamine induced hepatocarcinogenesis in rats. β-glucan powder was exposed to 50 KGY of gamma radiation for experimental study, healthy male rats were divided in five groups. Group 1: did not receive any treatment. Group 2: hepatic cancer group rats administrated with DEN orally at a dose of (20mg/kg b.wt/day) for six weeks. Group 3: Irradiated β-glucan group rats were orally administrated with Iβ- glucan for six weeks at a dose of 65 mg/kg body weight/day. Group 4: Protective group rats were administrated Iβ-glucan daily as in group 3 for two weeks they induced with DEN concomitant with Iβ-glucan for six weeks. Group 5: Therapeutic group rats were induced daily with DEN then administrated with Iβ-glucan for six weeks. DEN induced liver damage as evidence by a marked increase in serum indices of liver marker enzymes (Alt, Ast and GGT), tumor markers (alpha fetoprotein) and proinflammatory cytokines(interleukin-6) when compared with the normal control group. However, administration of irradiated β-glucan, in liver cancer induced rats displayed improvement in all measured parameters. In conclusion Iβ-glucan considered as a preventive and a therapeutic towards hepatocarcinogenesis via enhancing immune system in particular CD4, CD8 count and anti-inflammatory effect.

Keywords: Hepatocellular carcinoma, irradiated β-glucan, Tumor markers, β-catenin, caspase-3, CD4 and CD8.

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1. INTRODUCTION

β-glucans are one of the active compounds responsible for the immune effects of herbal products is in the form of complex polysaccharides, composed of D-glucobyransoyl exist inside the cell wall of many organism species such as mushrooms and yeasts (Brown and Gordon, 2005).

All β -glucans are glucose polymers linked together by 1 - 3 linear β-glyosidic chain core and they differ from each other by their length and branching structures (Stone et al., 1992).

depolymerization of β-glucan using ionizing radiation by the cleavage of the glycosidic bonds used simply as an effective method to produce low molecular weight β-glucans with high solubility and low viscosity, easily absorbed by intestinal mucosa without causing any notable changes in the functional-group status as compared with acidic or enzymatic hydrolysis (Byun et al., 2008; Choromanska et al., 2015). Most β-glucans pass to the proximal small intestine and some are coughed by macrophages (Sung et al., 2009; Noss et al., 2015). The targeted immune cells of β-glucans include macrophages, neutrophils, monocytes, natural killer (NK) cells, and dendritic cells (Yoon et al., 2008). Consequently, β-glucans can modulate both innate and adaptive responses, and they can enhance phagocytosis (Leung et al., 2006; Chan et al., 2009).

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer-related deaths worldwide (ZHU et al.,2016). The majority of HCC cases are attributable to underlying infections caused by hepatitis B and C viruses (Gupta et al., 2010). 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 (Waris et al., 2006). The objectives of the present investigation were to evaluate irradiated β-glucan on hepatocellular carcinoma.

2. MATERIALS AND METHODS:

Glucan preparation:

Glucan was extracted from dry mushroom, Agaricus bisporus, obtained from Ploshia (Giza, Egypt). The dry mushroom was added to 0.1 mol/L of NaOH and stirred for 30 min at 60°C. The material was heated to 115°C at 8.5 psi for 45 min and then allowed to settle for 72 hours. The sediment was suspended and washed with distilled water by centrifugation at 350 g for 20 min. The alkali-insoluble solids were combined with 0.1 mol/L acetic acid and heated to 85°C for 1 hour and 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 hours with periodic mixing. The material was centrifuged and the pellet was washed twice with distilled water followed by two washes with 100% acetone. The harvested solid material (glucan powder) was dispersed on drying trays and dried under vacuum at 38°C for 2 hours in the presence of Ca_2SO_4, and dried overnight under vacuum at room temperature.

Gamma irradiation:

Glucan powder was irradiated using Co-60 Gamma chamber 4000A at the National Center for Radiation Research and Technology, Atomic Energy Authority (Cairo, Egypt). β-glucan was exposed to a dose of 50 kGy at room temperature (25 ± 2°C). The source strength was approximately 11.1 PBq with a dose rate of 10 kGy/h. IGLU samples were stored at 4°C.

Animals:

Fifty 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±20C) 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 equal groups of 10 animals each, as follow:

- Groups I: rats kept as control animals.
- Group II: rats administrated with DEN orally at a dose of (20mg /kg b. wt/day) for six weeks for liver cancer induction (Nishikimi et al., 2006).
- Group III: rats were orally administrated daily with irradiated β-glucan (65 mg /kg b. wt/day) for six weeks (El-Sonbaty et al., 2013).
- Group IV: rats were administrated irradiated β-glucan daily as in group III for two weeks then experimentally induced with DEN concomitant with irradiated β-glucan for six weeks (protective group).
- Group V rats were experimentally induced daily with DEN as in group II then administrated with irradiated β-glucan for six weeks (therapeutic group).

At the end of 6 weeks, all animals were sacrificed 24 hours after the last treatment under light anesthesia. Blood samples were collected and serum was separated and stored at -20°C for subsequent analyses. A midline abdominal incision was performed in each animal and liver tissue specimens taken and divided in to two parts, one part was homogenates were used for the assessment of biochemical determinations caspase 3 and β-catenin. The other part was quickly rinsed with water and kept in 10% formalin for histopathological examination.

Biochemical analysis:

Serum alanine transferase ALT, ASTand GGT activities and creatinine level were determined using kits purchased from Biometry Co. (Egypt) according to the method described by Reitman and Frankel (1957), Dufour, (2010), Bartels et al. (1972), respectively. Also, serum alpha-fetoprotein (AFP), interleukin-6 (IL-6) and the activity of caspase-3 were determined by using the corresponding ELISA kit provided by Cusabio Biotech (China).

Molecular analysis:

a-Detection of β-catenin 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 β-catenin, 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 complementary DNA (cDNA) synthesis was performed with reverse transcriptase (Invitrogen) according to the manufacturer's protocol using 1µg of total RNA as the template.

. 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 gene are as follows, 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 (Pfaffl et al ., 2001). Each experiment was performed in triplicate in two independent experiments

b-Determination of CD4 and CD8 by flow cytometry:

The flow cytometer used for enumeration of T-cell subsets (CD4 and CD8) IS FACS caliber flow cytometer (Becton Dickinson, Sunnyvale, CA, USA) equipped with a compact air cooked low power 15 m watt argon iron laser beam (48nm). The average number of evaluated nuclei per specimen 20.000 and the number of nuclei scanned was 120/ second. CD4 and CD8 histograms were obtained with a computer program for Dean and Jett mathematical analysis (Dean et al., 1974).

Histological examination:

Liver samples were taken from rats in different groups and fixed in 10% formalin for 48 h. Tissues were washed by water then serial dilutions of alcohol (methyl-, ethyl-, and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin beeswax at 56°C in hot air oven for 24 h. Paraffin tissue blocks of 4 µm thickness were cut by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin and eosin (H&E) stain for routine examination with microscope (Banchroft et al ., 1996).

Statistical analyses:

All data were expressed as the mean ± standard deviation (SD). 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 to be statistically significant. All data were analyzed by Statistical Package for Social Science (SPSS) version 13 for Windows (SPSS, USA) software program.

3. RESULTS

Effect of irradiated β-glucans administrator on some biomarkers in DEN-induced liver cancer in rats. It was found that treatment of animals with DEN induced hepatic injury caused a significant increase in serum Alt, Ast and GGT activities along with increments in AFP level illustrated in figure (1,2,3 and 4) accompanied by significant increase in CD4, CD8, caspase 3 and IL-6 in comparison with both controls and irradiated β-glucans treated groups illustrated in figure (5,6,7 and 8) and a significant decrease in β-catenin and creatinine in comparison with control group illustrated in figure (9 and 10). Preventive and therapeutic administration of irradiated β-glucans showed statistically significant improvement in all measured parameters versus the DEN group.

Histopathological findings:

Liver tissues either for control or IGLU 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. 11A&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. 11 B1&2). With IGLU 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. 11D & E).

Table (1): Effect of administration of Irradiated B-glucan on serum (Alt , Ast , GGT , AFP , Creatinine) in hepatocellular carcinoma male albino rats (mean±S.E.):

Group / Parameter	ALT (U/L)	AST (U/L)	GGT (U/L)	AFP (ng/ml)	Creatinine (mg/dl)
Negative control	62.1 ± 1.01^{b}	64.8 ± 1.55	119 ± 1.32^{b}	0.86 ± 0.7^{b}	1 ± 0.05^{b}
positive control	213.6 ± 6.76^{a}	323.6 ± 3.41	291 ± 13.46^{a}	3.6 ± 0.24^{a}	1.4 ± 0.04^{a}
irradiated β -glucan group	67.6 ± 4.57^{b}	60.6 ± 3.78	109 ± 2.81^{b}	0.73 ± 0.05^{b}	1 ± 0.06^{b}
Protective group	180.8 ± 2.57^{ba}	491.5 ± 298.5^{a}	189 ± 5.34^{ba}	2.09 ± 0.01^{ba}	1.14 ± 0.08^{b}
HCC + irradiated β -glucan	203.7 ± 2.94^{a}	214.5 ± 5.34	205 ± 6.20^{ba}	2.65 ± 0.27^{ba}	1.2 ± 0.04^{ba}

S.E: Standard Error

a, b: Mean values with different superscript letters in the same row are significantly different at (P≤0.05).

Table (2): Effect of administration of Irradiated B-glucan on (Caspase-3, IL-6, CD4, CD8, B-catenin) in hepatocellular carcinoma male albino rats (mean±S.E.):

Group / Parameter	Caspase-3 (ng/gm protein)	IL-6 (pg/gm)	CD4 ( )	CD8 ( )	β -catenin ( )
Negative control	1.5 ± 0.08	18.95 ± 0.96^{b}	32.3 ± 0.97^{b}	30.4 ± 1.1^{b}	1.0 ± 0.01^{b}
positive control	2.2 ± 0.08	101.24 ± 2.55^{a}	59.9 ± 5.56^{a}	62.6 ± 1.6^{a}	5.7 ± 0.12^{a}
irradiated β -glucan group	2.25 ± 0.12	13.25 ± 0.34^{ba}	33.4 ± 1.34^{b}	33.1 ± 1.7^{b}	1.0 ± 0.01^{b}
Protective group	6.4 ± 0.43^{ba}	59.4 ± 2.87^{ba}	45.3 ± 1.49^{ba}	46.5 ± 2.4^{b}	2.9 ± 0.15^{ba}
HCC + irradiated β -glucan	8.3 ± 0.64^{ba}	55.8 ± 1.6^{ba}	44.2 ± 1.5^{ba}	50.0 ± 0.95^{ba}	2.4 ± 0.19^{ba}

S.E: Standard Error

a, b: Mean values with different superscript letters in the same row are significantly different at (P≤0.05).

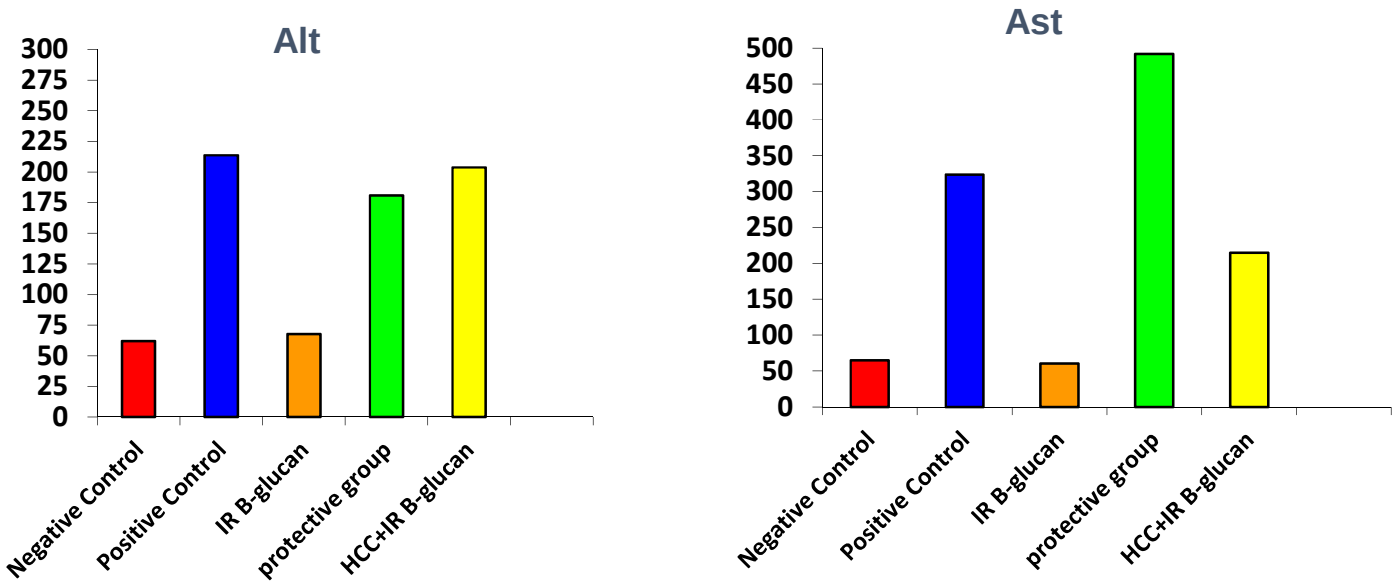

Fig. (1,2): Alt and Ast levels in different treatments compared with controls. Each column represents mean±SE (n=6). a P<0.05 compared to control; b P<0.05 compared to DEN group.

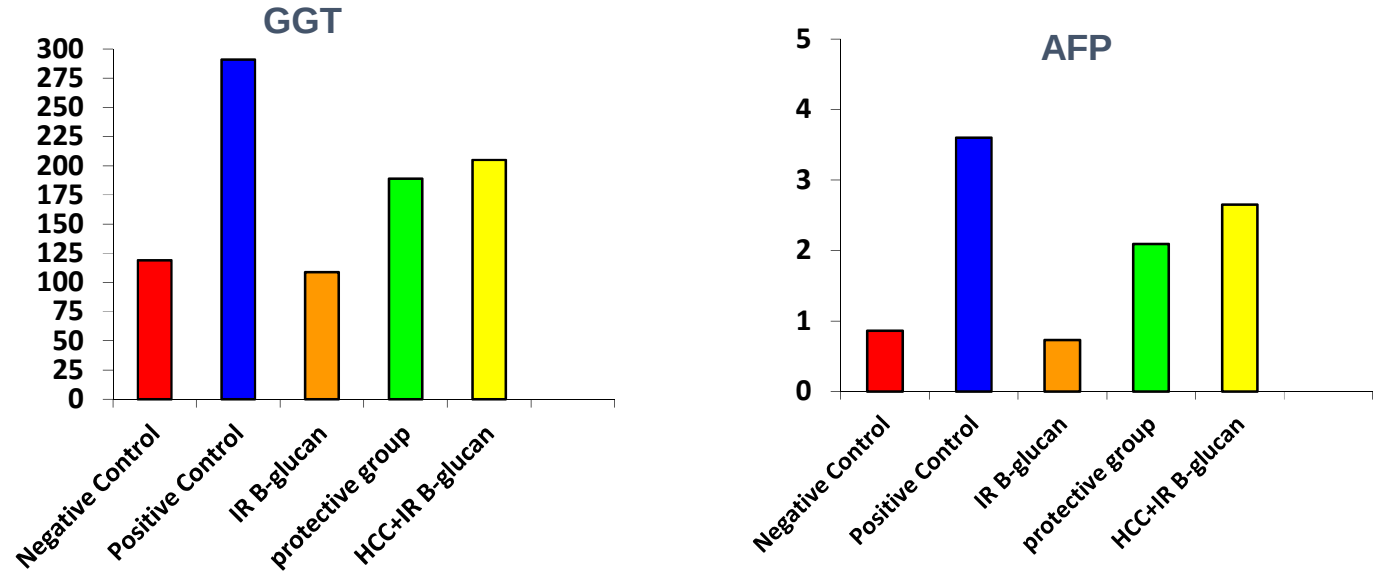

Fig. (3,4): GGT and AFP levels in different treatments compared with controls. Each column represents mean±SE (n=6). a P<0.05 compared to control; b P<0.05 compared to DEN group.

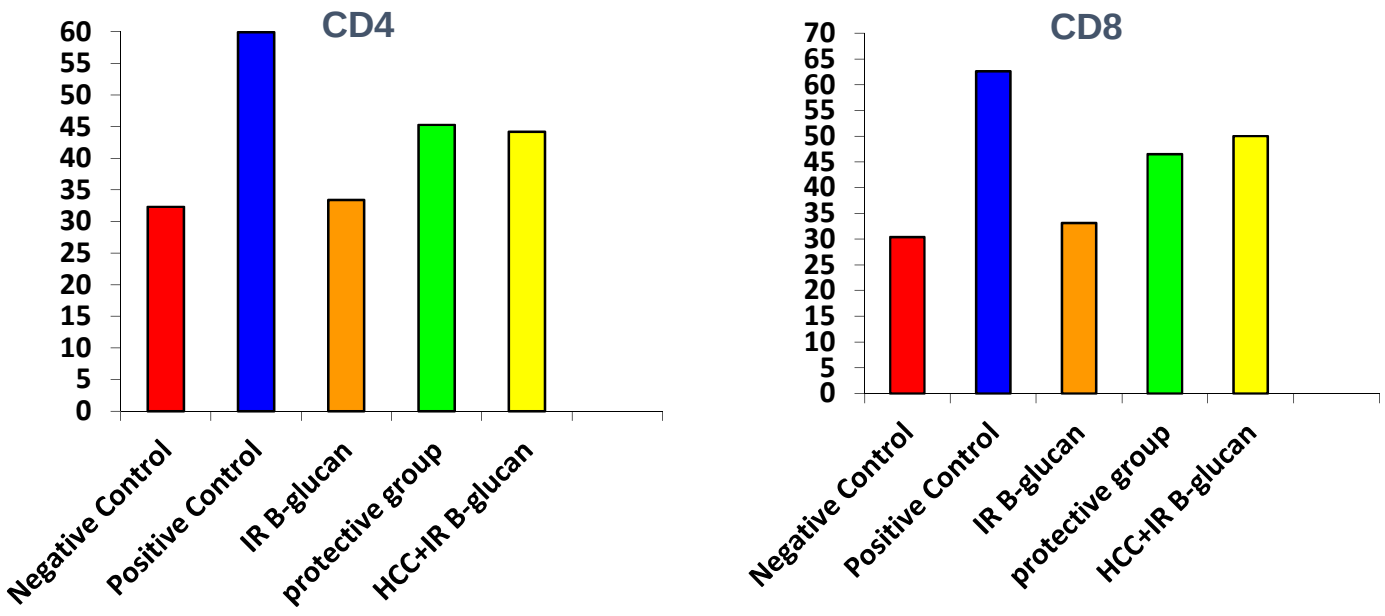

Fig. (5,6): CD4 and CD8 levels in different treatments compared with controls. Each column represents mean±SE (n=6). a P<0.05 compared to control; b P<0.05 compared to DEN group.

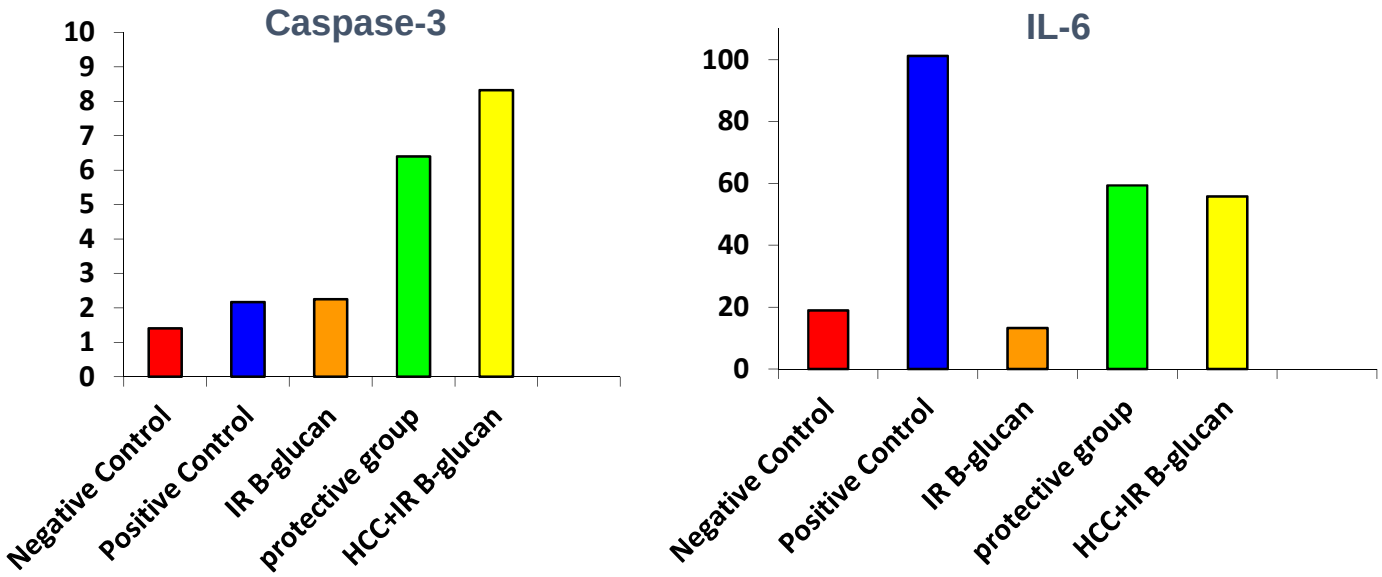

Fig. (7,8): caspase-3 and IL-6 levels in different treatments compared with controls. Each column represents mean±SE (n=6). a P<0.05 compared to control; b P<0.05 compared to DEN group.

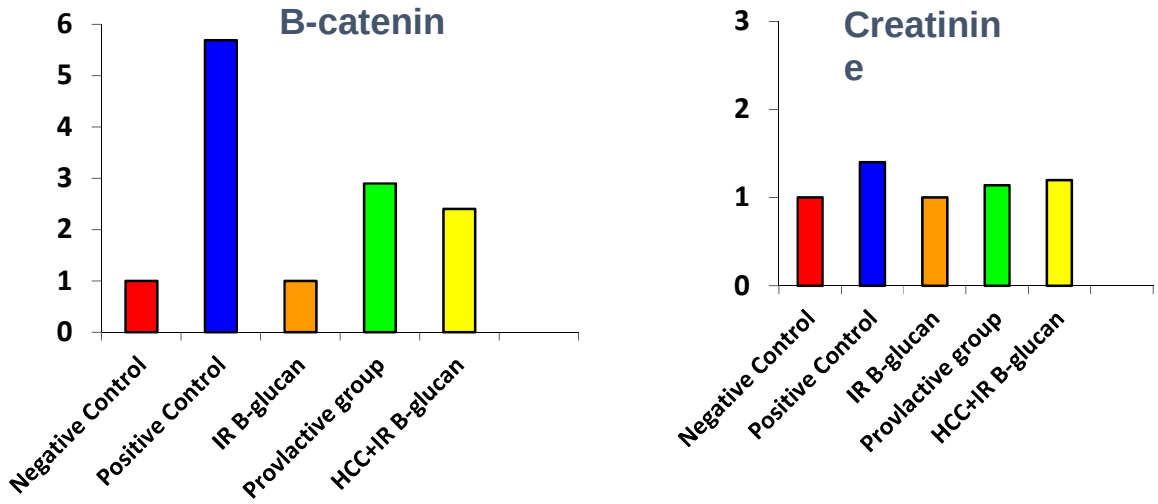

Fig. (9,10): β -catenin and creatinine levels in different treatments compared with controls. Each column represents mean±SE (n=6). a P<0.05 compared to control; b P<0.05 compared to DEN group.

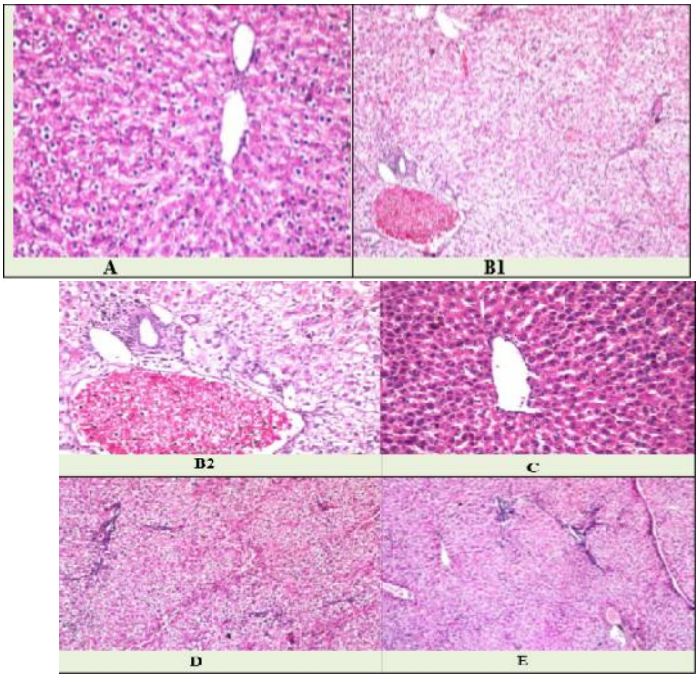

Fig. 11: Photomicrographs of liver specimens stained with hematoxylin and eosin showing
(A) Normal hepatic architecture in control animals. (B1) The portal area with inflammatory cells infiltration and congestion in portal vein in DEN group. (B2) Fibrosis in-between the degenerated dysplastic hepatocytes in DEN treated rats. (C) Normal hepatic architecture in GLU treatment.(D) Fibrosis in portal area as well as in-between the degenerated dysplastic hepatocytes in protective group. (E) Fibrosis in portal area and in-between the degenerated dysplastic hepatocytes in therapeutic group.

4. 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 chemo preventive 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 (Hussein et al ., 2014). 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 (Byun et al., 2016; Byun et al., 2008). It is known that serum ALT, AST and GGT activities are indicative for hepatic function; their increase is correlated with the hepatic injury (Zhao et al., 2014) 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, the obtained data showed that, stimulation of liver with DEN leads to tissue damage as was established by the elevated ALT, AST and GGT activities. The decrease of serum ALT, AST and GGT activities in irradiated β-Glucan treated rats may be attributed to the decrease of cellular damage. Tumor marker like AFP is a potential screening tool 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 (Merrick et al., 2006). The current study indicated a significant increase in serum levels of AFP of DEN-treated rats. Upon treatment with irradiated β-glucans, the serum levels of the tumor marker; AFP was significantly declined. The mechanisms of irradiated β-Glucan 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 irradiated β-Glucan. However, previous study was done on HepG2 cells, irradiated β-Glucan 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 irradiated β-Glucan in cancer therapy (Piresa et al., 2013).DEN stimulation causes a release of pro-inflammatory marker; IL-6 level and 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 (Lane et al., 2011). also, induction of tumor angiogenesis (Rabinovich et al., 2011). Additionally, a novel role of IL-6 signaling identified in assisting NF-KB signaling to synergistically induce the transcription of proinflammatory genes. It was named the “inflammation amplifier (Ogura et al., 2008). Caspase 3 is one of large 14 members plays an important role in apoptosis (program cell death). In this investigation, the obtained data showed that levels of caspase 3 was

significantly lowered with DEN treatment. In the treatments using only irradiated β-Glucans (protective and therapeutic) of irradiated β-Glucan, the value of caspase 3 was significantly higher than that in the DEN-treated animals. This result demonstrates that the β-glucan administrator had a protective effect.β-Catenin has an essential role in intercellular adhesion and signal transduction. β-catenin functions as a transcriptional activator downstream in the Wnt signal-ling pathway. Cytoplasmic stabilisation of β-catenin, mainly due to inactivating mutations of the adenomatous polyposis coli (APC) tumour suppressor gene or activating mutations in exon 3 of the β-catenin gene, can activate this important pathway in the development of several carcinomas. Also, β-catenin, the vertebrate homologue of armadillo protein in Drosophila, is a multifunctional protein involved in two apparently independent processes: cell–cell adhesion and signal transduction (Peifer et al., 1995). Recently, it has been demonstrated that cytoplasmic β-catenin participates in the transduction of wingless Wnt signals and activates transcription by forming complexes with DNA-binding proteins (Molenaar et al., 1997). Intracellular concentrations of β-catenin are mainly regulated by degradation, which is probably initiated by interaction with the adenomatous polyposis coli protein (APC) (Ilyas et al., 1997). and phosphorylation on serine or threonine residues of codons (Rubinfeld et al., 1997). The APC tumor suppressor gene has frequently been found to be mutated in human colorectal adenomas and colon carcinomas but rarely in other malignancies, such as esophageal carcinomas (Powell et al., 1997). The mutant APC proteins found in colon carcinomas result in β-catenin stabilization and a significant increase of this protein within the cell which may then activate β-catenin/Tcf signaling (Korinek et al., 1997). In the same manner, dominant activating β-catenin mutations that render the protein insensitive to APC/GSK-3β-mediated degradation could lead to a dysregulation of the signaling function of β-catenin and thus to carcinogenesis (Morin et al., 1997). Oncogenic activation of β-catenin by amino acid substitutions or interstitial deletions has been reported in colorectal (Takahashi et al., 1998). and melanoma (Rubinfeld et al., 1997). cell lines, hepatocellular carcinomas (Koch et al., 1999).CD4 & CD8: CD4 cells (T-helper cells) and CD8 cells (killer T cells) are types of white blood cell that fights infections, made in the spleen, lymph nodes and thymus gland. β-glucan acting as immunomodulating effect on both innate and adaptive response by enhancing T-cells, activation of CD4 and CD8 T-cells and increase the number of specific CD8 via receptors on macrophages and neutrophils (Wang et al., 2010).

5. CONCLUSION

The obtained findings showed that, irradiated β-Glucan ameliorated hepatic damage induced by DEN which contributed to experimental hepatocarcino-genesis in rats. irradiated β-Glucan is considered as a promising treatment toward hepatocarcinogenesis.

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