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The Usage of ß-glucan Extracted from Local Mushroom as Immunomodulator

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Abstract: The present study was conducted to investigate the active compound of local cultured mushroom (β-glucan), and studied it effect immunologically on some organs (liver, spleen, small intestine). The results of drying 1 kg of fresh mushroom was 80 g dried material, the carbohydrates (polysaccharides) were extracted from the dried powder of mushroom by ethanol (99%).The carbohydrate and protein concentration were 88, 1.12 mg/mL, respectively, then the purification method was done for the extracted material, which were separated in to three main peaks, the carbohydrate concentration measured which were 490, 405 and 315 mg/mL, respectively, while Lowry method was detected the protein concentration for these peaks which were 0.0038, 0.0032 and 0.0023 mg/mL, respectively. HPLC carried out to identify β-glucan for these peaks. The results showed that the first peak was close one to standard β-glucan. The three peaks were examined as immunomodulator for lab. Animals (mice) that were divided into three groups according to the peaks of ion exchange diagram plus group of control. The results of immunization method showed crossly enlargement of liver, splean and small intestine, for all experimental animals groups, and microscopically changes in the tissues structure for these organs, generally there were infiltration of mononuclear cells in all these organs especially for the first group.

Key words: β-glucan, ion exchange, HPLC, immunomodulator, local mushroom, polysaccharide

INTRODUCTION

β-glucans-(1-3), (1-6) which is a branched glucose polymers derived from the cell wall of a variety of plants and microorganisms such as barley, yeast and mushroom (Suzuki *et al*.,1990; morikawa *et al*., 1989), β-glucan is a immuno stimulant exert pleiotropic activation of the innate immunity in mice and humans(Di-Renzo, 1990; Abel and Czop,1992). Activation of macrophages, neutrophil granulocytes and Natural Killer (NK) cells by β-glucans leads to elevated phagocytic activities and production of reactive oxygen intermediates and pro inflammatory cytokines *in vitro* and *in vivo* (Seljelid *et al*., 1989; Liang *et al*., 1998).

Cells of the innate immunity have surface beta-glucan receptors, which specifically recognize and bind the beta-1,3-glucan linkage of the beta-glucan molecule, Lymphocytes belong to the acquired immunity and play a key role in defending the body against disease (Xiao *et al*., 2004).

Bacterial or fungal products can initiate the immune response mostly by binding to the innate immune receptors like lectin receptors (mannose receptor, Dectin-1) (Underhill *et al*., 2005). Recently, showed that beta glucan interacts on cell surface with dectin-1 receptors for its biological effects. Dectin-1 receptors are expressed mainly on macrophages, neutrophils, dendritic cells, and a subpopulation of T-lymphocytes (Brown *et al*., 2003).

The goal of this study was to determine the effects of extractable β-glucans obtained from local mushroom as immuno stimulating agent for leukocytes when used as *in vivo* stimulants in mice.

MATERIALS AND METHODS

This study has been conducted during November 2008 to August 2009, in Biotechnology Department, Baghdad university .

This procedure was done according to (Yap and Ng, 2001). Locally cultured and purchased Fruit bodies of mushroom (1 kg) was dried and grinded into powder.

Extraction of polysaccharides from mushrooms: The two hundred fifty gram of dried powder was mixed with 700 mL of boiled water at 100ºC for 1 h. The sample was cooled and added equal volume of ethanol absolute. The mixture was centrifuged at 3000 rpm/min for 10 min under cooling 4ºC. The pellet was boiled in hot water for 10 min and cooled, and centrifuged at 6000 rpm/min for 15 min. under cooling 4ºC.

Equal volume of 95% ethanol was add to the supernatant, and left for 18 h at 4ºC, then centrifuged at 6000 rpm/min for 15 min. under cooling 4ºC. Then the pellet was recovered after centrifugation ,and dissolved in PBS buffer and dialyzed against tap water for 3 days at 4ºC with changed the distilled water every day.

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Primary purification: The yield from previous step was taken and added equal volume of triacetic acid 20%. The suspension was filtrated by filter paper (Whitman no 1), and washed the precipitant on filter paper with ethanol 98% with three fold as filtrated solution. The solution was centrifuged at 3000 rpm/min for 10 min. Under cooling 4ºC. Then the pellet was removed and dissolved with distilled water and dialyzed (against distilled water) for 3 days with changed the distilled water every day.

Secondary purification: More purification process was done by Ion exchange (DEAE) to obtain as possible pure yield. The column dimension was (60×2.5) cm, the washed and eluted buffer was Tris HCl (15 mM) pH8.4.

Carbohydrate analysis: Carbohydrate content was determined by the phenol - sulfuric acid method (Dubois *et al*., 1956).

Carbohydrate standard curve preparation: Standard curve for carbohydrate was prepared by using different concentration of glucose (20, 40, 60, 80, 100 μg/mL)

Protein analysis: Protein content was determined by the Bradford method (Bradford, 1976).

Protein standard curve preparation: standard curve for carbohydrate was prepared by using different concentration of bovine serum albumin (20, 40, 60, 80, 100 μg/mL).

Fraction collection: According to the dispersed peaks of Ion exchange, the fractions were collected in to three groups (16-34), (35-49), (50-58) respectively, then concentrated by dialysis sacs against sucrose.

High performance liquid chromatographic quantization of β-glucan: The samples and standard of b-glucan were analyzed by HPLC separation with column Luna 5u C_{18} (250×4.6) mm internal diameter (id). The mobile phase was acetonitrile (ACN) 100% with a flow rate of o.5 ml/min. Injection volume for sample and standard solution was 10 μL. The pH was adjusted to 3.5. The detection occurred at UV light at 305 nm wave length.

Invivo experiment: Twenty four mice were divided into 4 groups, each one contain 6 mice. One of this group was control, while other groups were divided according to the peaks of Ion exchange curve.

Immunization procedure: This procedure was done according to the method described by (Naohito *et al*., 2001). Each animals group unless control was injected with 500 μg/mL of each dispersed peaks intraperitonally. After 1 week, each animals group was injected intraperitonally again with the same dose. The animalswere scarified after one week of the last dose. Some organs (liver Small and Large intestine, Spleen) were taken and preserved with 10% formalin.

Histological section: Histological section was done for liver, spleen and intestine.

RESULTS AND DISCUSSION

The result of drying 1 kgof fresh mushroom was 80 g dried material, so the percentage of the solid material was 8%, while the percentage of the water was 92%. On the other hand, the result of extraction method showed the conc. of protein and carbohydrate material was 1.12 and 88 mg/mL, respectively.

The purification result by using ion exchange (DEAE), showed, dispersion three peaks in washing fractions. The first one between tube number (16-34), the second (35-49) and the third one was (50-58), as shown in Fig. 1. These peaks (Fig. 1) were classified to three groups. The protein concentration for these groups were 0.0038, 0.0032 and 0.0023 mg/mL, while the carbohydrate concentration were 490, 405 and 315 mg/mL, respectively (Fig. 2).

The using of HPLC analysis to measure the degree of carbohydrate purity, the results showed the purification method efficiency, specially for the first group (Fig. 3), the retention time were appeared, two peaks, the first was 4.102, and the second was 5.882 and when compared with the retention time of the standard β-glucan (Fig. 4), so the second peak is represented the β-glucan because of closely related with that of standard curve. While the second group retention time (Fig. 5) showed seven peaks 0.272, 0.525, 1.034, 2.781, 3.587, 3.994 and 5.953, respectively.

This result showed several impurities in the sample, but the last peak is represented the b- glucan when compared with standard sample. The last group analysis showed (Fig. 6) there are three retention times (peaks), which are 2.554, 5.643 and 5.974, respectively. The last two retention time showed closely related with standard β-glucan and about the tissues studied for animal's organs, there are crossly enlargement in liver and spleen in all groups and also there are microscopically changes when compared with the control one.

The First conc. Liver showing normal structure appearance with kupffer cell hyperplasia (Fig. 7a). The intestine showing normal structure with mononucleus (macrophage, monocyte, lymphocyte, plasma cell) infiltration, infiltrate inside the villi (Fig. 7b). Spleen follicular hyperplasia of the white pulp (Fig. 7c), with

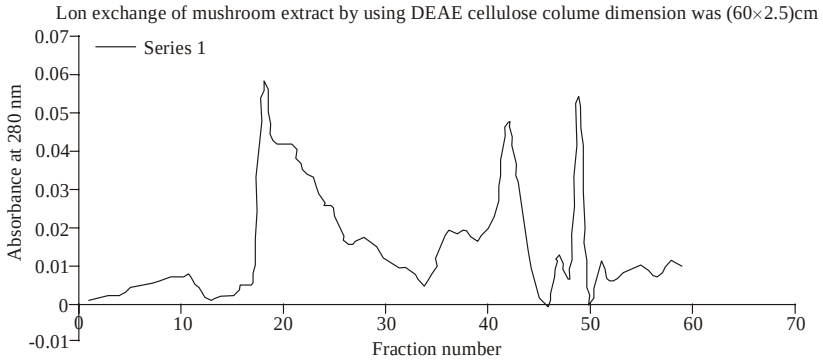


Fig. 1: Protein measurement by using Ion exchange DEAE cellulose

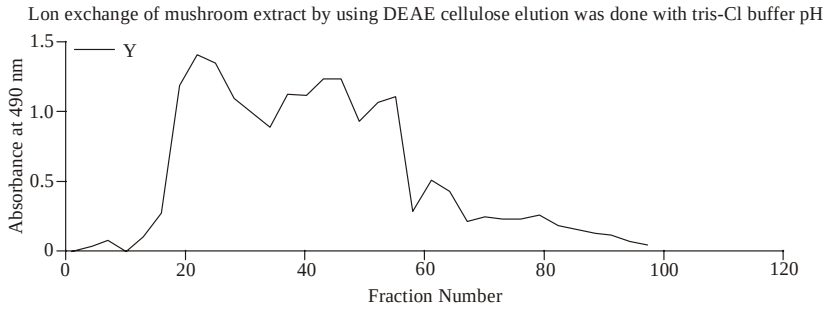


Fig. 2: Carbohydrate measurement by using Ion exchange DEAE cellulose

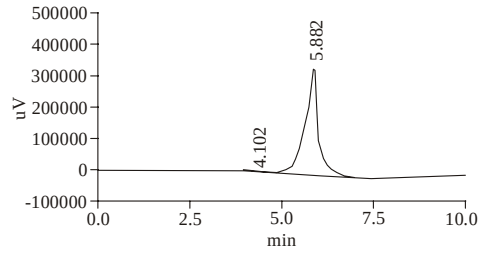


Fig. 3: HPLC analysis for first peak

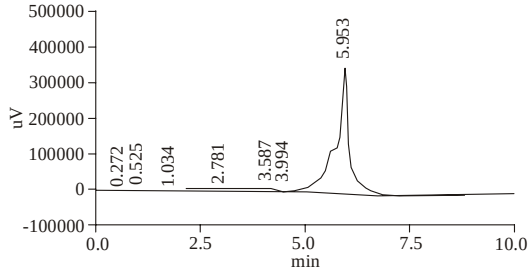


Fig. 5: HPLC analysis for second peak

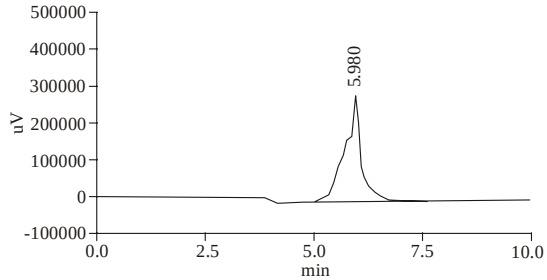


Fig. 4: HPLC standard curve for β-glucan

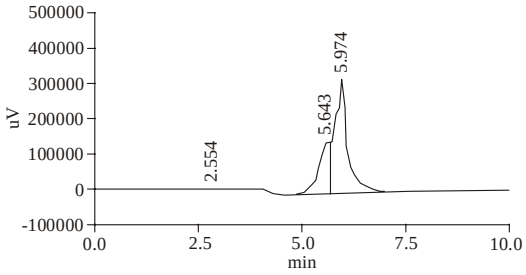
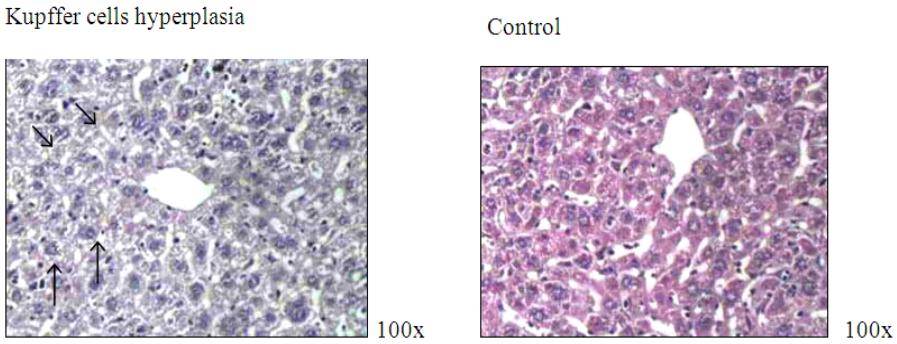
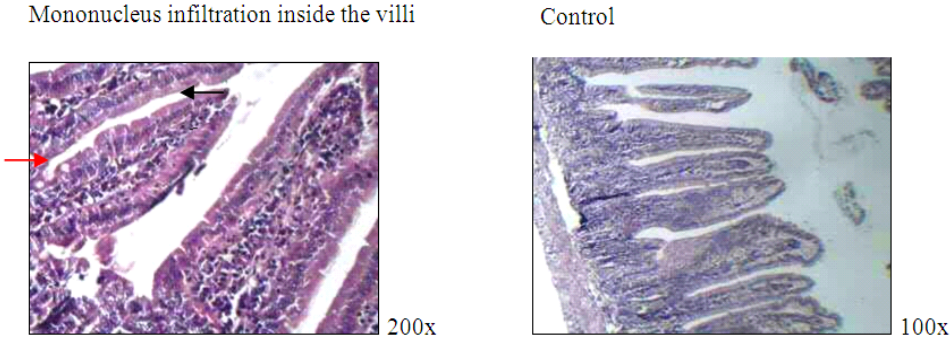


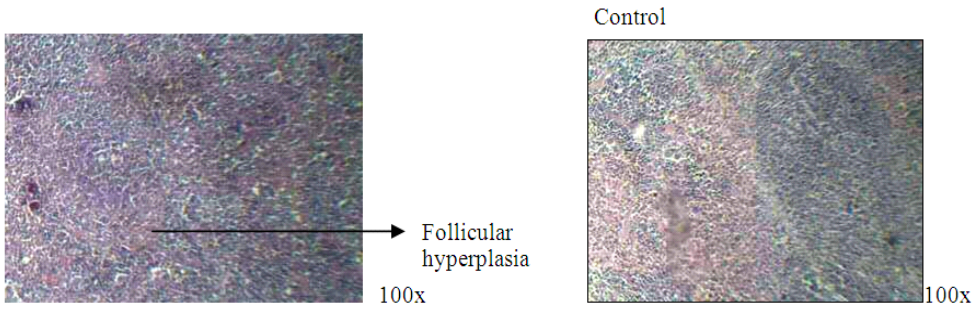
Fig. 6: HPLC analysis for third peak



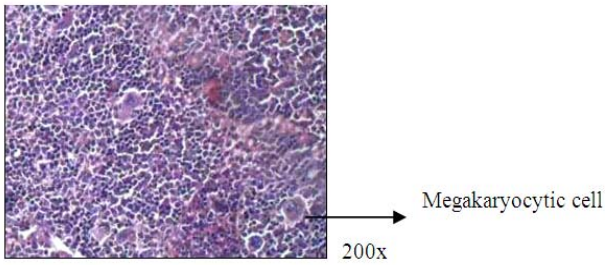
(a) kupffer cell hyperplasia Third Third conc.conc.



(b) Mononucleus (macrophage, monocyte, lymphocyte, plasma cell) infiltration, infiltrate inside the villi

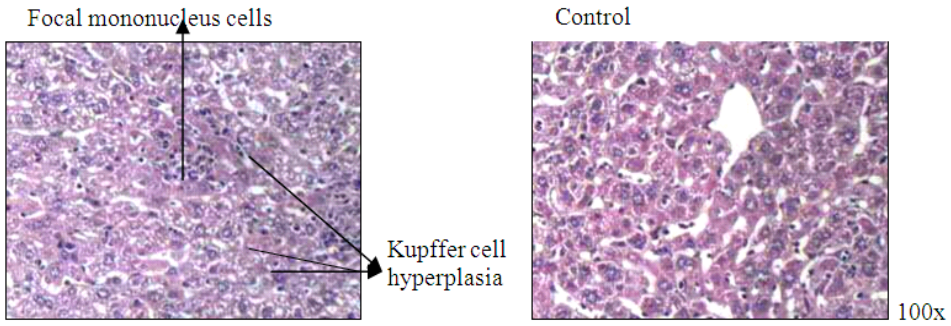


(c) Spleen follicular hyperplasia of the white pulp

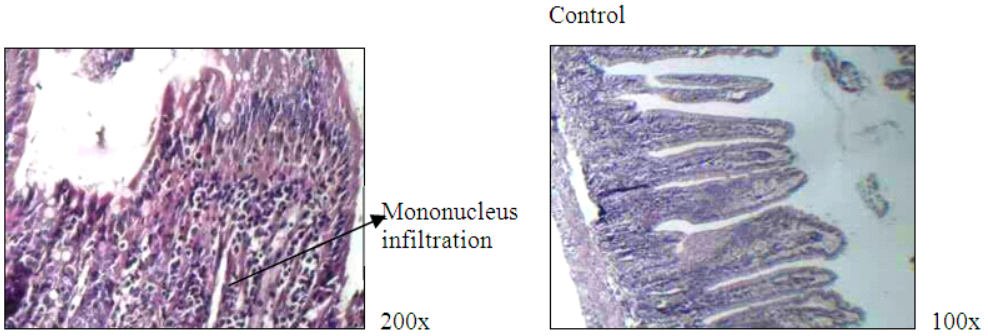


(d) megakaryocytic cells infiltrate in the spleen tissue

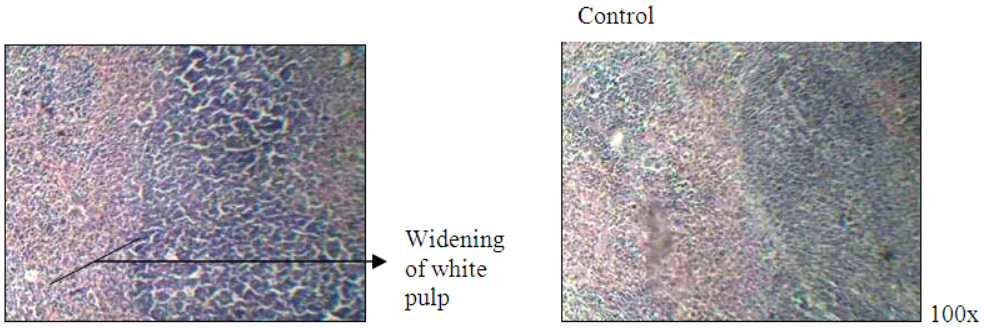
Fig. 7: First conc. Liver showing



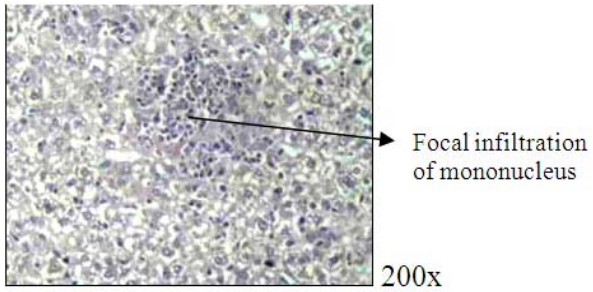
(a) focal mononucleus cells



(b) mononucleus cell infiltrate inside the villi

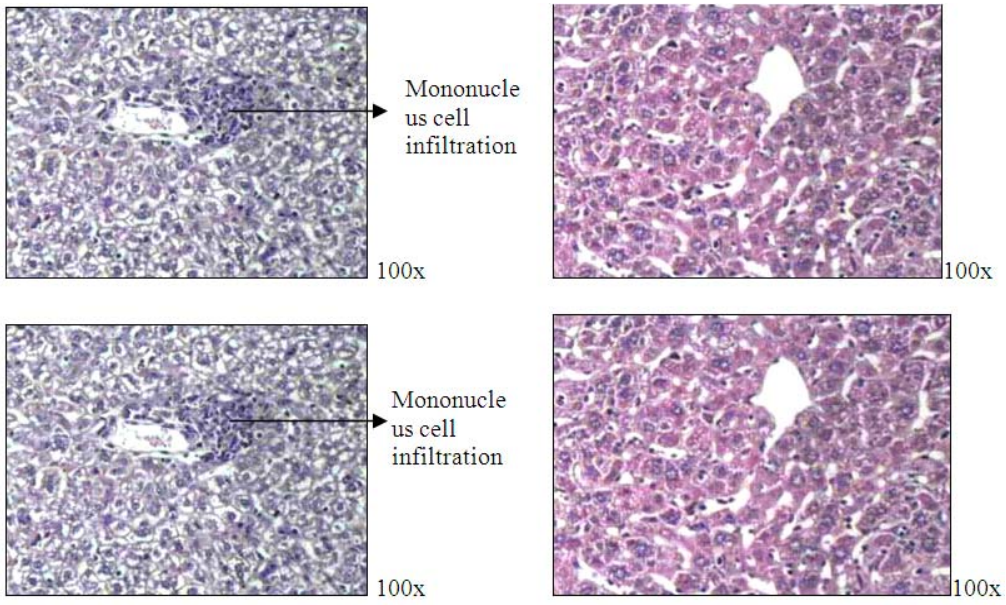


(c) diffuse hyperplasia with widening of white pulp

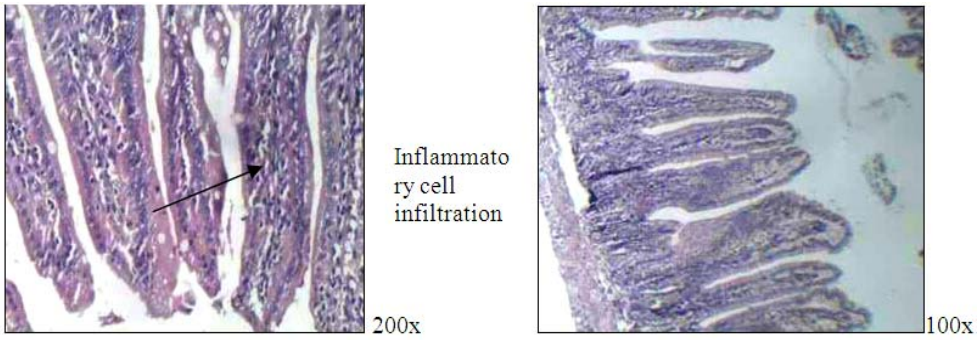


(d) focal infiltration of mononucleus cell

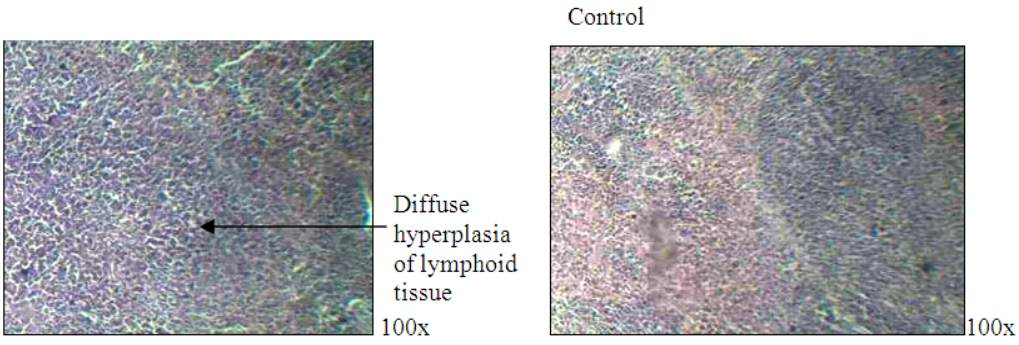
Fig. 8: Second conc.



(a) infiltration of mononucleus cell



(b) infiltration of inflammatory cell



(c) diffuse hyperplasia of parenchyma tissue

Fig. 9: Third conc.

megakaryocytic cells infiltrate in the spleen tissue (Fig. 7d). The second conc. Liver tissue showing normal with focal mononucleus cells infiltrate (Fig. 8a) and the intestine prominent mononucleus cell infiltrate inside the villi (Fig. 8b), While in spleen there is diffuse hyperplasia with widening of white pulp (Fig. 8c), focal infiltration of mononucleus cell (Fig. 8d) and the third conc. Liver showing infiltration of mononucleus cell (Fig. 9a) around central vein and focal infiltration for the same cell in the tissue of liver and mild degeneration. Intestine normal structure of villi with mild inflammatory cells infiltrate inside the villi (Fig. 9b) and the spleen showing diffuse hyperplasia of parenchyma tissue (lymphoid tissue) (Fig. 9c).

From this study we can concluded ,the extraction method was efficient in extracted beta-glucan from local mushroom , and there was a significant result in the yield of carbohydrate when compared with global research, and the *in vivo* study showed, all peaks fractions have positive effects in animals immune system but the first one is the best ,and the appearance in different peaks may be due to the different types of β-glucan (1-3), (1-4),(1-5),(1-6) that may be appeared each one alone or in two types, or three types, or in whole form due to the extraction method.

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