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ANTI-ANGIOGENIC EFFECT OF β-GLUCAN EXTRACTED FROM *PLEUROTUS SPP*

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ABSTRACT

This study was conducted to investigate the effect of β-glucan extracted from the medicinal mushroom *pleurotus eryngii* as an anti-angiogenic and anti-tumor factor. The β-glucan was extracted from the mushroom *Pleurotus eryngii* using hot water extraction method. The extracted glucan was confirmed by both HPLC and FT-IR which were used to analyze the chemical structure. Carbohydrates and proteins contents were determined for the glucan and the results indicated that the carbohydrate content was very high and protein content was low. The anti-angiogenic effect of the extracted β-glucan was evaluated using CAM assay that was carried out using fertilized eggs with age of 8 days, different doses of β-glucan were used and in comparison with the negative control. Results showed that high doses showed a decreased number of blood vessels in the fertilized eggs as compared with the negative control which indicated the inhibition of fertilized egg neovascularization, while the low dose showed no significant effect on the neovascularization. The anti-tumor effect of β-glucan was evaluated in mice were subcutaneously inoculated with 5 $\times 10^5$ AN3 adenocarcinomas cells line at from thigh toward the shoulder region. After the tumor size attained 5 mm in the average major axis. Different doses of β-glucan were intraperitoneally injected in tumor bearing mice for ten consecutive days. Histopathological sectioning revealed both necrosis and an increased number of inflammatory cells in the treated tumors as compared with the negative control.

INTRODUCTION

Medicinal mushrooms have been used in traditional Oriental therapies for their antitumor and immunomodulating properties.[1] Studies have shown that edible mushrooms, which demonstrate medicinal or functional properties, include species of the genera *Lentinus, Hericium, Grifola, Flammulina, Pleurotus, and Tremella.*[2] These mushrooms were reported

to have therapeutic activities specially *Pleurotus* spp. which occurs throughout the hardwood forests of the world that include the most diverse climates.[3] The dry matter of different mushroom fruit bodies is about 5–15%, they have a very low fat content and contain 19–35% proteins. Mushroom fruit bodies are plentiful of vitamins, mainly B1, B2, C and D2.[4] The content of carbohydrates, which are mainly present as polysaccharides or glycoproteins, ranges 50–90%. Most abundant mushroom polysaccharides are chitin, hemicelluloses, β and α-glucans, mannans, xylans and galactans. The average molecular mass Mw of them varies according to the source and ranges from 5 to 2000 kDa.[5] These Polysaccharides are believed to be the bioactive ingredients involved in antitumor and anti-inflammatory effects.[6] Mushroom polysaccharides are present mostly as linear and branched glucans with different types of glycosidic linkages, such as (1 - 3), (1 - 6) β-glucans and (1-3) α-glucans, but some are true heteroglycans containing glucuronic acid, xylose, galactose, mannose, arabinose or ribose.[7] Many researches and studies on the importance of β-glucan were highly concentrated on their biological activity and their interaction as non-adaptive immunomodulating agent and other biological effects when applied in different systems in *vivo* and in *vitro.*[8] Basically, those studies revealed that β-glucan is clarified for its property to enhance immune system. In addition, beta-glucan, shows strong efficacy for activating macrophages and neutrophils. β-glucan activated macrophages or neutrophils can recognize and kill abnormal cells including abnormal cancerous cells or antiangiogenic activities.[9] Besides to the immunological effects of β-glucan, many studies proved that β-glucan stimulates phagocytic activity as well as synthesis and release of interleukin-1(IL-1), IL-2, IL-4, IL-6, IL-8, IL-13, and tumor necrosis factor-alpha,[10] inhibitory effects on the growth of tumor cells *in vivo* and affects expression of several important genes in tumor cells,[11] cell cycle arrest and induction of apoptosis can be seen,[12] activated complement receptors on natural killer cells, neutrophils, and lymphocytes may also be associated with the detected tumor cytotoxicity,[8] and finally anti-angiogenic properties.[13] Angiogenesis is a dynamic process of endothelial proliferation and differentiation. Tumors with high angiogenic activity have been correlated with poor patient survival.[14] The major physiological stimuli for angiogenesis include inflammation and tissue ischemia and hypoxia. A number of specific factors are known to stimulate angiogenesis, including vascular endothelial growth factors (VEGFs), inflammatory cytokines, and adhesion molecules.[15] In general, oxidative stress is a common central theme of inflammation and tumor growth. During tumor growth, increased reactive oxygen species (ROS) activate tumor-infiltrating leukocytes to induce an angiogenic

response.[16] Application of angiogenesis inhibitors is relatively less toxic than conventional chemotherapy and has a lower risk of drug resistance.

METHOD

Extraction β-Glucan from *Pleurotus eryngii*

β- glucan was extracted from *Pleurotus eryngii* collected from from Ministry of Agriculture\Department of Organic Farming in Baghdad using hot water extraction method:[17] The dried mushroom fruit bodies were powdered using a blender for 5 minutes. The powdered mushroom (50 g) was mixed with 1 L of distilled water in a ratio of (1:20). The pH of the mixture was adjusted to 7 with 20% Na2Co3. The mixture was heated to 90°C for 6 hrs using shaker water bath. The mixture was centrifuged at 8000 rpm for 10 min at 4°C. The pellet was discarded and the supernatant was taken.

The pH of the supernatant was adjusted to 4.5 with 2M HCl to precipitate protiens. Centrifuge at 8000 rpm for 30 min at 4°C. The pellet was discarded and the supernatant was taken. Ethanol absolute was added to the supernatant in a ratio of (1:1) and left for 12 hrs at 4°C to precipitate the β-glucan. The suspension then was centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was taken. Then the pellet was homogenized with ethanol absolute and then dried by oven at 60°C.

Analysis of β-glucan by High Performance Liquid Chromatography (HPLC) Technique

The samples and standard of β-glucan were analyzed by HPLC with column Lichrosphere C18 (4.6 mm x 50 mm), 3 mm particle size. Mobile phase: deionized water. Detection: refractive index detector RF Shimadzu, Flow rate: 1.2 ml\min Temperature: 30°C.

Preparation of sample: 10 mg were dissolved in 250 ml to get 40 µg\ml standard, And then 20 µl were injected into HPLC column for analysis. The separation occurred on liquid chromatography Shimadzu 10AV-LC equipped with binary delivery pump model LC-10A Shimadzu, The eluted peaks were monitored by UV-Vis 10 A-SPD spectrophotometer.

Analysis of β-glucan by FT-IR (Fourier Transformed Infrared)

The chemical structure of β-glucan from *pleurotus* spp. was analyzed by Fourier Transformed-Infrared spectrometry (Shimadzu IR Affinity – Japan) at the Chemistry Department /College of Science /AL-Nahrain University. The FTIR spectrum (an advanced infrared (IR) spectrometry) was utilized to detect the functional groups of glucan structure

compared with the standard. This was done under FTIR spectrometry in the wavelength ranged of 400-4000 cm^{-1} and at a resolution of 8 cm^{-1}. This test involved mixing an equal volume of glucan sample and standard glucan with potassium bromide (KBr), then the mixture was analyzed by the FTIR analyzer.

Determination of Carbohydrate Concentration

Carbohydrate concentration was determined by phenol - H_2SO_4 method.[18]

Estimation of Protein Concentration

Protein concentration was determined according to Bradford.[19]

Chick Chorioallantoic Membrane (CAM) Assay for the β-Glucan

Ex vivo anti-angiogenic activity of glucan was measured by CAM assay.[20] A group of 10-7 days-old fertilized eggs was incubated at 37.5 ◦C with 55% relative humidity. On the eighth day, a $1cm^2$ window was carefully created on the broad side of the egg shell, which can candle the egg to assure existence of embryonic blood vessels. A volume of glucan containing at differernt doses and normal saline (as control) were applied on a filter paper disk and then placed into CAM, after which a permeable sticky tape was immediately appended to the window. After incubation for 3 days (until day 11), the egg shell was pushed aside around the window, and the blood vessels were photographed. Anti-angiogenic effect of glucan on CAMs was quantified by counting the number of blood vessel branch points which were marked using artistic software on the photos.

Tumor Induction

According to the procedure, twenty inbred Albino Swiss females mice, aged 6-8 weeks, 20-25 g in weight were supplied by Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) animal house unite. The animals were subcutaneously inoculated with 5×10^5 AN3 adenocarcinomas cells line from thigh toward the shoulder region. The animals were housed in a plastic cage containing hard-wood chip as bedding. The housing conditions followed the guidelines of the ICCMGR. Ahmed Majeed 2003 (AM3) also named (AN3) mammary adenocarcinoma transplantable tumor line (2.1.6) was supplied from ICCMGR, Experimental Therapy Department. Single tumor (mammary adenocarcinoma) bearing mouse, was used to obtain tumor cells which were later transplanted into adult female albino mice.[21] The following protocol was followed to perform the transplantation process,[22] which was done under sterile conditions:

The tumor mass region well disinfected with 70% ethyl alcohol. By using 10ml disposable syringes, the contents of tumor mass tissue withdraw into sterile flask. The solid content was allowed to settle down while the supernatant discarded. The residues washed 2-3 times with sterile PBS by final wash, appropriate amount of PBS was stayed. This amount was comparable with the number of animals which prepared to transplantation. Generally, the withdrawing content from tumor mass of single mouse was adequate for transplantation of on average 10 mice. Separations of the tumor material into cells suspension made through mechanical disaggregation of cells in the withdrawing materials, by vigorous pipetting (withdraws and return of contents several times). Each adult female albino mice (6-8 wk. old) became ready to tumor cell transplantation; 0.1ml of tumor cell suspension $5x10^5$ was transplanted through insertion of a needle (gage No.18) subcutaneously from thigh region toward the shoulder region where the injection was performed.

Tumor Treatment with β-Glucan

This experiment was conducted to evaluate the effect of β glucan as an anti-tumor factor. The experimental mice were divided into four groups, three groups of mice were intraperitoneally injected with β-glucan suspended in physiological saline at different doses for 10 consecutive days while the vehicle-control mice received an equivalent volume of physiological saline. At the tenth day, the mice were sacrificed, tumor samples were collected and preserved in formalin (10%) for histological sectioning.[23]

Histological Analysis of Tumors

Sections were first isolated and cleaned from access adipose tissues in a petridish containing normal saline then the sections were weighted by an electronic precision balance then the sections were put in tubes containing 10% formalin for about 16-18 hours for fixation purpose, then they were transferred in to tubes containing 70% ethanol alcohol in which they preserved till the time of final preparation. In the final preparation the samples were transferred in 99% alcohol for about 6 hours then put in xylol for 2 hours.[24] Then sections were put in a paraffin wax for blocks preparation. Then a 5 µ paraffin sections were obtained using a sharp knife of a handling laboratory microtome. Paraffin sections of tissue were put in a water bath of 43 ºC then were stick in a glass slide in a proper angled manner to obtain the best result, then glass slides containg sections were dried using hot plate. For all histological slides, pictures were taken using a digital camera under light microscope at a magnification

power of 200X and 400X. The staining method was performed using heamatotoxilin and eosin stain.[25]

RESULTS AND DISCUSSION

β- Glucan by HPLC

In this study, this technique was used to determine the quality and purity of *Pleurotus eryngii* β-glucan, in addition to confirm the structural similarity with the standard β-glucan. The HPLC analysis revealed a major peak (2.087) of a liquid glucan (Figure 1.a), which indicating the presence of the extracted glucan. Such peak showed the same retention time of the glucan standard (2.193) (Figure 1.b).

The HPLC was used for the separation of the components of β-glucan extracted from mushrooms that gave an efficient method for detecting the β-glucan.[26]

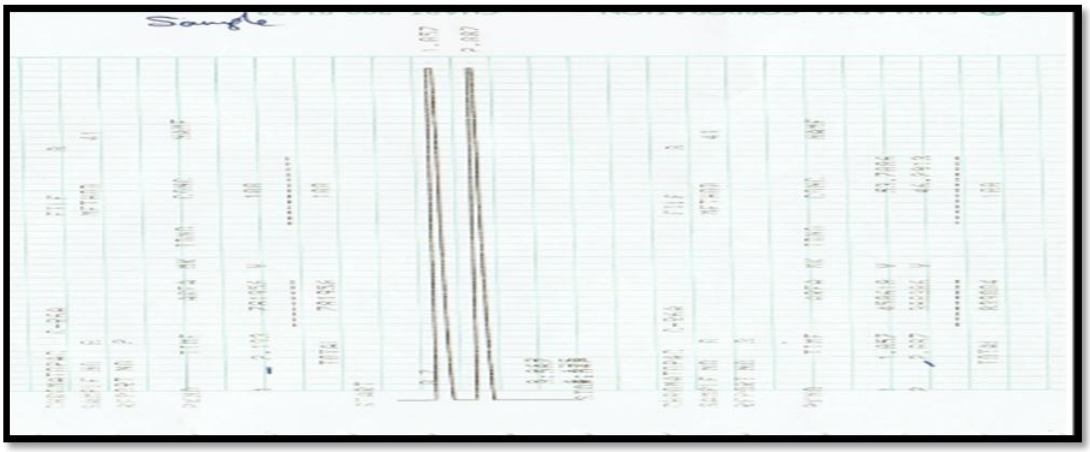

Figure 1(a): HPLC analysis for extracted *P. eryngii* β-glucan.

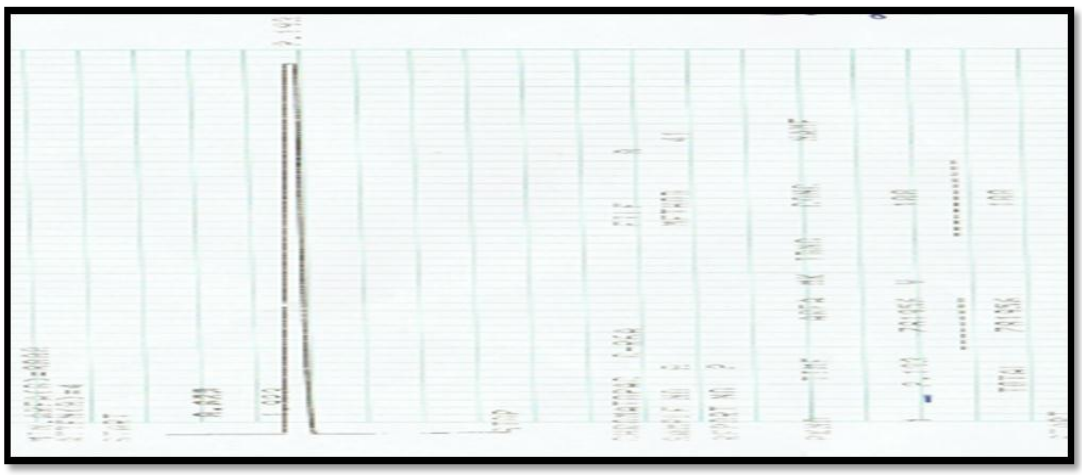

Figure: 1(b): HPLC analysis for standard *P. eryngii* β-glucan.

β-glucan by FT-IR

Glucan was analyzed using FT-IR spectroscopy to detect the functional group in its chemical structure of glucan, and compared these groups with standard ones. FT-IR is one of the most widely used methods to identify the chemical constituents and elucidate the compounds structures and has been used as a requisite method to identify medicines in pharmacopoeia of many countries. Owing to the fingerprint characters and extensive applicability to the samples, FT-IR has played an important role in pharmaceutical analysis in recent years.[27] Result in Figure (2.a) showed that infrared spectrum at the absorbance (1028.0 cm^{-1}) means the presence of C-O-C bonds which is a characteristic feature for β-glucan structure stretching with the standard (1055.0 cm^{-1}) (Figure 2.b).[28]

Figure 2(a): FT-IR analysis for extracted *P. eryngii* β- glucan.

The absorbance at (1371.3 cm^{-1}) refers to the presence of C-H aliphatic bending; the standard absorbance was at (1315.4 cm^{-1}).[29] On the other hand, free hydroxyl groups and carboxyl groups were absorbed at regions (2927.7 cm^{-1}) and (2922.0 cm-1) that found in the carbohydrate.[30] Results indicated that the FT-IR spectra of the extracted glucan has appearance typical to that of the standard β-glucan with high degree of purity and absence of the protein contents that absorbed at 1635, 1542, 1650 cm^{-1}.[31]

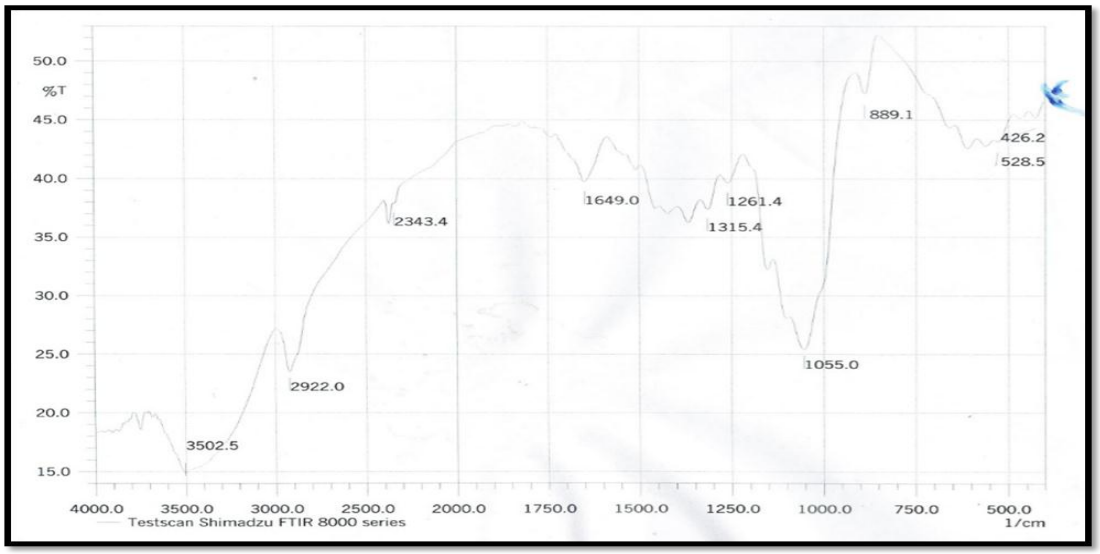

Figure 2(b): FT-IR analysis for standard P. *eryngii* βeta-glucan.

Carbohydrate and Protein Content in extracted β-glucan

Chemical composition of the glucan extracted from *P. eryngii* was characterized by estimating the carbohydrate contents according to Dubois *et al.* depending on the standard curve of glucose, and estimating the protein contents according to Bradford, depending on the standard curve of bovine serum albumin. Accordingly, The components of both glucose and protein gave an important indication about glucan purity due to the high amount of sugars with low content of proteins. Wasser reported that the content of carbohydrates, which are mainly present as polysaccharides or glycoproteins, ranges at 50–90%; the most abundant polysaccharides are chitin, α- and β-glucans, and hemicelluloses (e.g. mannans, xylans, and galactans). The polysaccharides in mushrooms are present mostly as glucans with different types of glycosidic linkages, such as branched (1→3), (1→6)-β-glucans and linear (1→3)-α-glucans, but some are true heteroglycans. the glucan purity is an important character in determination of its application since glucan may be used in pharmaceutical, cosmetics, food and other felids.[32]

Anti-angiogenic effect of *P. eryngii* β-Glucan

The anti-angiogenic effect of the *P. eryngii* β-glucan was detected by CAM assay. According to the results, the concentration 250µg\egg of β-glucan showed a non-significant inhibition effect on neovascularization as compared with the negative control (61.0±2.08) and the number of blood vessels branches was (58.33±1.45) as shown in figure (3 and 4 A,B). While the concentrations (500, 1000 µg\egg) showed a significant inhibition of the neovascularization as compared with the negative control and revealed a decreased number of

blood vessels branches (51.67±1.76 and 45.67±0.33) as shown in figure (3 and 4 C,D). On the other hand, it was noticed that when the concentration of β-glucan is increased to 2000µg\egg, this will result in a significant decrease of CAM capillaries (37.67±1.76) as shown in figure (Figure 3 and 4 E). This result revealed that the increase in the concentration of β-glucan will decrease the chick CAM angiogenesis.

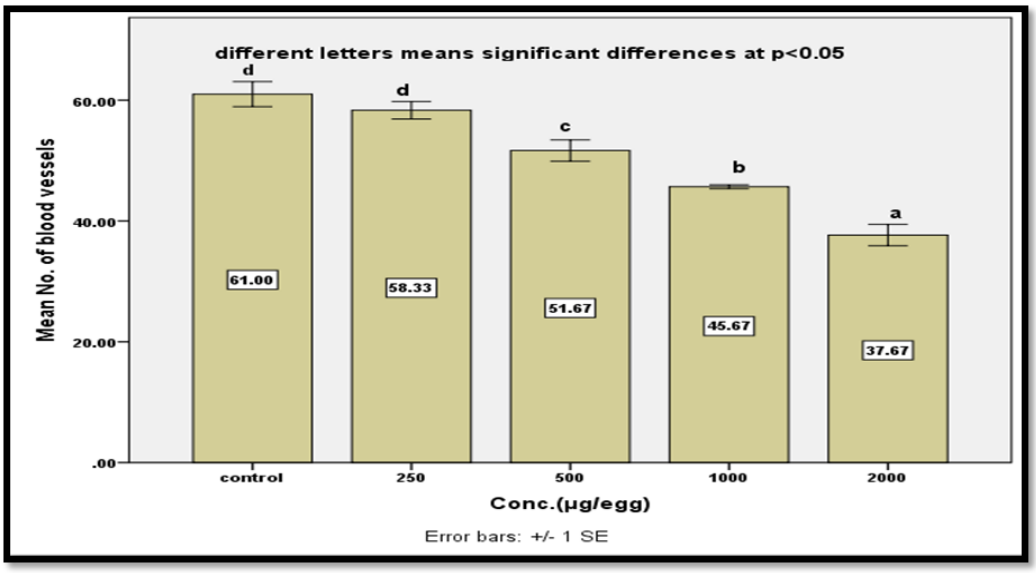

Figure 3: Effect of β-glucan on the neovascularization of the chick embryo.

That regulates the formation of blood vessels; it was found that β-glucan extracted from *L. edodes* (medicinal mushroom) inhibited angiogenesis by suppressing VEGF expression, leading to slow progression of tumors.[35]

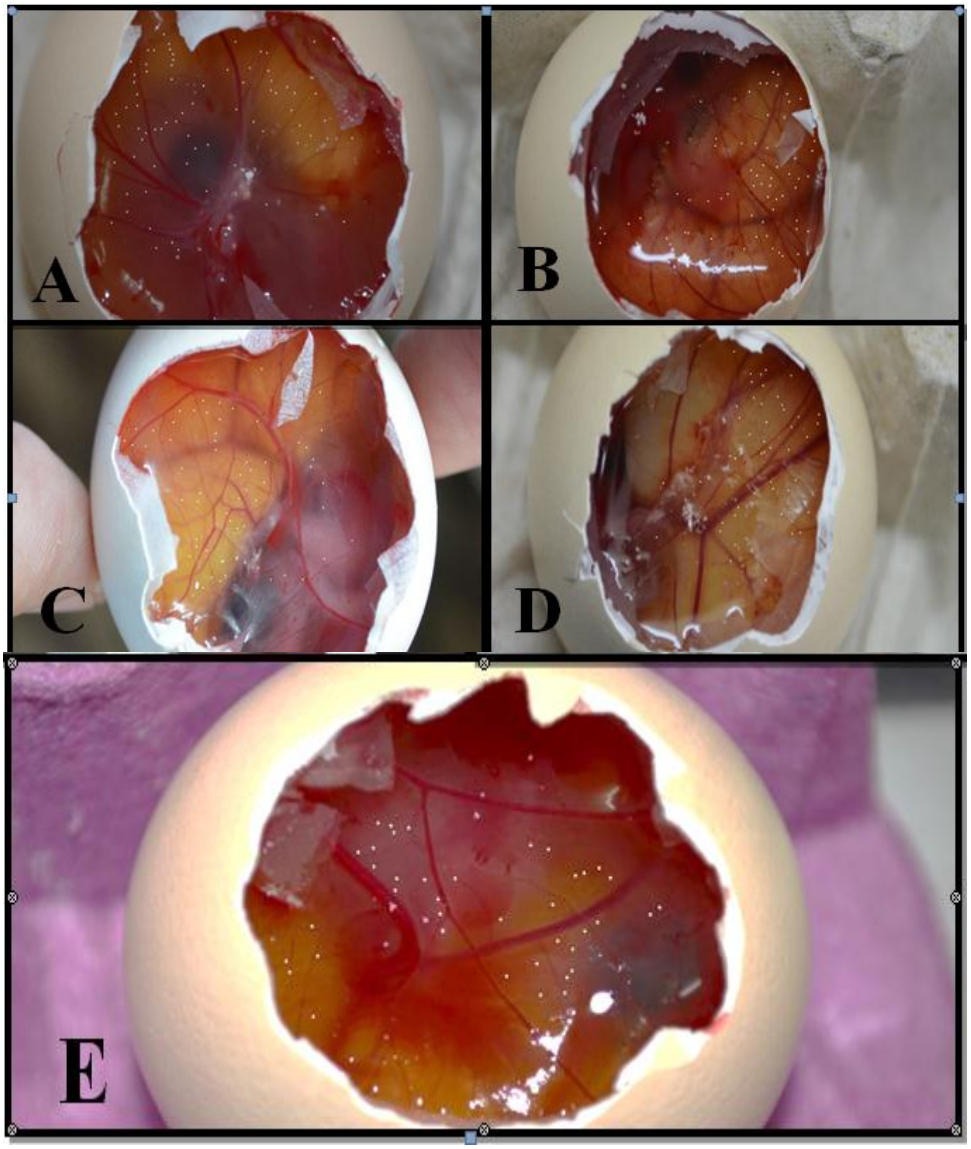

Figure 4: The effect of *P. eryngii* β-glucan concentrations on the neovascularization of the chick embryo blood vessels.

A-Control, B-250µg\egg, C-500µg\egg,

D-1000µg\egg E-2000µg\egg

(The dotes in the figure indicates the number of blood vessels branches.)

Anti-Tumor Effect of *P. eryngii* β-Glucan in AN3 murine adenocarcinoma mice

Different histopathological changes were observed in tumors sections obtained from mice bearing AN3 murine mammary adenocarcinoma and treated with different doses of β-glucan and control. Tumor section of normal mice control showing pleomorphic malignant cells which arranged as glandular structure (adenocarcinoma) (figure 5).

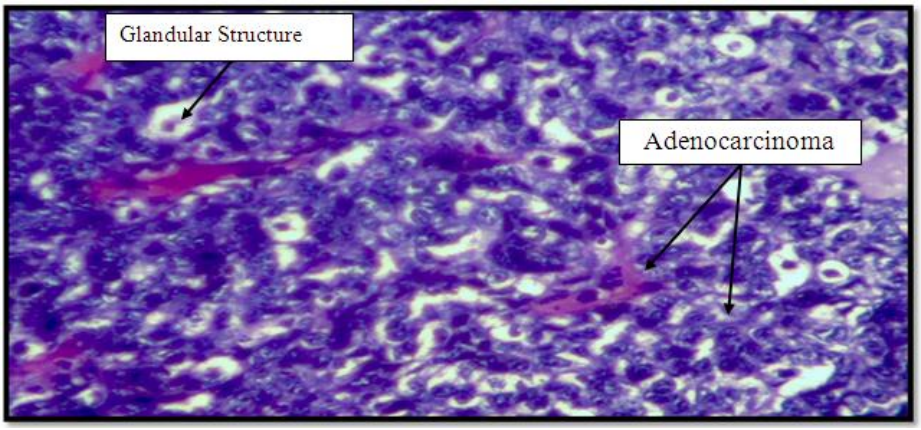

Figure 5: Tumor section of mice (control) showing pleomorphic malignant cells which are arranged as glandular structure (adenocarcinoma) (H and E; 400X).

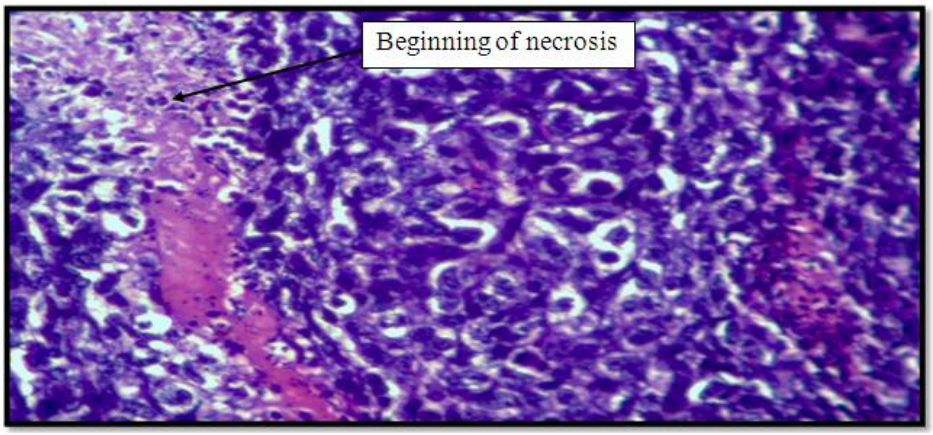

Figure 6: Tumor section of AN3 murine adenocarcinoma mice treated intraperitoneally with (200 mg\kg) extracted β-glucan showing a beginning necrosis of the malignant cells (H and E; 400X).

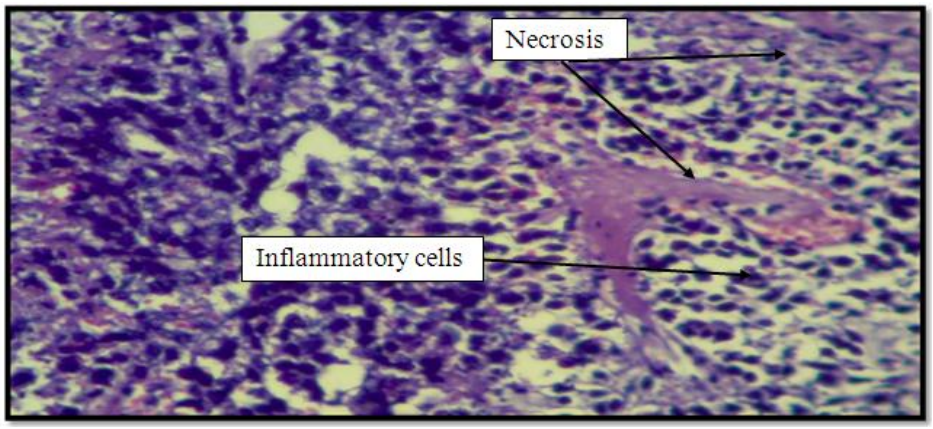

Figure 7: Tumor section of AN3 murine adenocarcinoma mice treated intraperitoneally with (200 mg\kg) extracted β-glucan mice (400 mg\kg) showing wide area of necrosis and inflammatory cells (H and E; 400X).

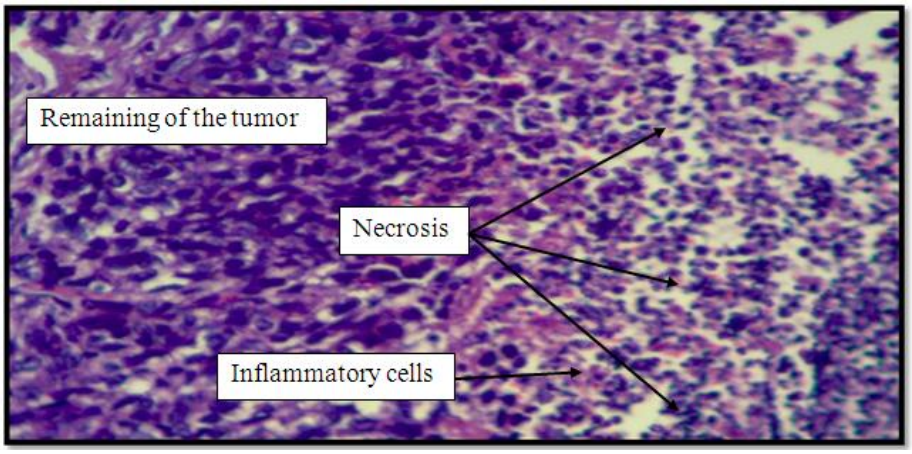

Figure 8: Tumor section of AN3 murine adenocarcinoma mice treated intraperitoneally with (800 mg\kg) extracted β-glucan showing inflammatory cells and more prominent appearance of necrosis of malignant cells (H and E; 400X).

The histopathological results indicated that the β-glucan extracted from medicinal mushroom *pleurotus eryngii* have induced the immune system and increased the activity of inflammatory cells which contributes probably to attenuated tumor growth in tumor-bearing mice. Many studies have shown that β-glucan exerts immune-stimulating effects. Namely, it functions through binding to CR3 (CD11b/CD18) of neutrophils, macrophages, and NK cells for cytotoxicity of iC3b-opsonized tumor cells.[23] *Sparassis crispa* (Hanabiratake in Japanese), is an edible and medicinal mushroom containing large amount of β-1,3-D-glucan. SC is known to show antitumor activity in oral administration. β- 1,3-D-glucan of SC is reported to act as an antitumor agent in tumor-bearing mice when injected intraperitoneally.[13] Recent studies have revealed that the levels of serum IL-2 and TNF- in Renal cancer-bearing mice were significantly increased by *P. eryngii* polysaccharide treatment. The increase of IL-2 and TNF- also explain the antitumorigenic properties of *P. eryngii* polysaccharide.[36] β-glucan affects several pathways in the immune and non-immune systems. For example, it can induce cytokines and nitric oxide production in macrophages and promote monocyte adhesion and activate neutrophils and natural killer cells combat against cancer cells.[37] In addition, experimental findings strongly demonstrated that β-glucan extracted from *L. edodes* (Lentinan) showed significant anti-tumor effects without toxicity through activating immune responses to inhibit tumor cell proliferation and angiogenesis, and to induce tumor cell apoptosis *in vivo*. Lentinan promoted cell apoptosis through caspase 3-dependent signaling pathway, and inhibited cell proliferation possibly through targeting p53 via enhancement of p21. Additionally, Lentinan inhibited Stat 3

phosphorylation in tumor tissues, possibly leading to suppression of cell proliferation and angiogenesis. These provide a strong rationale for using β-glucan to enhance treatment efficacy of cancer.[35]

REFERENCES

1. Liu F.; Ooi V.E. and Fung M.C. Analysis of immunomodulating cytokine mRNAs in the mouse induced by mushroom polysaccharides, Life Sci, 1999; 64: 1005–1011.
2. Kues, U., and Liu, Y. Fruiting body production in basidiomycetes, Applied microbiology and biotechnology, 2000; 54: 141-152.
3. Rosado, F. R., Carbonero, E. R., Kemmelmeier, C., Tischer, C. A., & Iacomini, M. A partially 3-O-methylated (1 fi 4)-linked a-D-galactan and a-D-mannan from *Pleurotus ostreatoroseus* Sing. FEMS Microbiology Letters, 212, 261–265 Medicinal Mushrooms, 2002; 1: 69–80.
4. Manzi, P., Gambelli, L., Marconi, S., Vivanti, V., & Pizzoferrato, L. Nutrients in edible mushrooms: an inter-species comparative study. Food Chemistry, 1999; 65: 477–482.
5. Bohn, J. A., & BeMiller, J. N. (1 - 3)-β-D-glucans as biological response. Carbohydrate Polymers, 1995; 28: 3–14.
6. Wang S.Y.; Hsu M.L.; Hsu H.C., Tzeng C.H., Lee S.S.; Shiao M.S. and Ho C.K. The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes, Int. J. Cancer, 1997; 70: 699–705.
7. Wasser S.P. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Applied Microbiology and Biotechnology, 2002; 60: 258–274.
8. Chen, J. and Seviour, R. Medicinal importance of fungal beta-(1-3), (1-6) glucans. Mycol. Res, 2007; 111(6): 635-652.
9. Mayell, M. and Maitake, A. Extracts and their therapeutic potential. Altern. Med. Rev, 2001; 6(1): 48-60.
10. Pelley, R. and Strickland, F. Plants, polysaccharides, and the treatment and prevention of neoplasia. Crit. Rev.Oncog, 2000; 11(34): 189-225.
11. Mantovani, M.; Bellini, M. F.; Angeli, J. F.; Oliveira, R. J.; Silva, A. F. and Ribeiro, L. R. β-Glucans in promoting health: Presentation against mutation and cancer. Mutation Rese, 2008; 658(3): 154-161.

12. Zhang, M.; Chiu, L. C.; Cheung, P. C. and Ooi, V. E. Growth inhibitory effects of a beta-glucan from the mycelium of *Poriacocos* on human breast carcinoma MCF-7 cells: cell cycle arrest and apoptosis induction. Oncol. Rep, 2006; 15(3): 637-643.
13. Yamamoto A., Kimura T., Sugitachi A., and Matsuura N. Anti-angiogenic and Anti-metastatic Effects of b-1,3-D-Glucan Purified from Hanabiratake, *Sparassis crispa*. Biol. Pharm. Bull, 2009; 32(2): 259-263.
14. Koukourakis M.I., Giatromanolaki A., Fountzilas G., Sivridis E., Gatter K.C. and Harris A.L. Angiogenesis, thymidine phosphorylase, and resistance of squamous cell head and neck cancer to cytotoxic and radiation therapy. Clin. Cancer Res, 2000; 6: 381–389.
15. Ziche M., Donnini S., and Morbidelli L. Development of new drugs in angiogenesis. Curr. Drug Targets, 2004; 5: 485–493.
16. Shono T., Ono M., Izumi H., Jimi S.I., Matsushima K., Okamoto T., Kohno K., and Kuwano M. Involvement of the transcription factor NF-kappa B in tubular morphogenesis of human microvascular endothelial cells by oxidative stress. Mol. Cell Biol, 1996; 16: 4231–4239.
17. Wood P.J. Physicochemical characteristics and physiological properties of oat(1-3)(1-4)-β-glucan. *Oat Bran*. Wood P.J., ed. St. Paul, MN: Amer. Assoc. Cereal Chem, 1993; 83-112.
18. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A. and Smith,F. Colorimetric methods for determination of sugars and related substance. Anal. Chem, Experimental Microbiology. 1st edition. Mosby-Year Book, Inc. USA, 1956; 28: 350-356.
19. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Annu. Biochem, 1976; 72: 248-254.
20. Li, M. H.; Miao, Z. H.; Tan, W. F.; Yue, J. M.; Zhang, X. W. and Ding, J. Pseudolaric acid B inhibits angiogenesis and reuces hypoxia- inducible factor 1 by promoting proteasome-mediated degration. Clinical Cancer Research, 2004; 10: 8266-8274.
21. Al-Shammari, A., Yaseen, Y. and Alwan, M.J. Establishment and characterization of AN3 first murine mammary adenocarcinoma transplantable tumor line in Iraq. Iraqi Journal of Cancer, 2008; 1(2): 1.
22. Al-Shammari, A.M., Yaseen, N.Y. and Alwan, M.J. Immunology Study for NDV Treatment in Mice Bearing Mammary Adenocarcinoma Tumor. Iraqi Journal of Cancer and Medical Genetics, 2011; 4(1): 11.

23. Yeun-hwa Gu, Yasuyuki Takagi, Takashi Nakamura, Takeo Hasegawa, Ikukatsu Suzuki, Masami Oshima, Hitoshi Tawaraya, and Yoshimi Niwano. Journal of Medicinal Food, 2005; 8(2): 154-158. doi:10.1089/jmf.2005.8.154.
24. Bankroft, L. Basic histological techniques; 4th ed; an Arboer science publishers, 1980; 130-145.
25. Junqueria, L. and Carneiro, J.R.O. Male Reproductive System. In: Basic Histology. Janson, M.; Harriet and Petery J., Stamford. 11th ed., 2005; 418-434.
26. Al-Aubydi, M. and Abed, R. M. The usage of β-glucan extracted from local mushroom as immunomodulator. Curr. Res. J. Biol. Sci, 2011; 3(5): 535-541.
27. Vlachos, N., Skopelitis, Y., Psaroudaki, M., Konstantinidou, V., Chatzilazarou, A., Tegou, E. Application of fourier transform infrared Spectroscopy to edible oils. Analyt chim Acta, 2006; 573: 459-465.
28. Hozova, B., Kuniak, L., Moravckov, P., and GajdoŠov, A. Determination of water-insoluble β-d-glucan in the whole- grain cereals and pseudocereals. Czech J. Food Sci., 2007; 25: 316-324.
29. Karreman, R. J., Dague, E., Gaboriaud, F., Guiles, F., DuvalJ. F., and Lindsey,G. G. The stress response protein Hsp12p increase the flexibility of the yeast *Sacchromycescerevisiae*. Biochim. Biophys. Acta, 2006; 1436: 239-246.
30. Ibrahim, M.; Alaam, M.; EI-Haes, H., Jalbout, J. F.; and Leon, A. Analysis of the structure and vibrational spectra of glucose and fructose. Ecl. Quim. Sao Paulo, 2006; 31(3): 15-21.
31. McCann, M. C. Hammouri, M. Wilson, R. Belton, P., and Roberts, K. Fourier Transform Infrared Microspectroscopy Is a New Way to look at Plant Cell Walls. Plant Physiol., 100:1940-1947.mice treated with a carcinogen, N-butyl N-butanol nitroso amine. Immunopharmacology and Immunotoxicology, 1992; 19: 175–183.
32. Vetvicka, V. and Vetvickova, J. An evaluation of the immunological activities of commercially available b-(1,3) glucans. JANA, 2007; 10: 25-31.
33. Del Vecchio, M., Bajetta, E., Canova, S., Lotze, M.T., Wesa, A., Parmiani, G. and Anichini, A., Interleukin-12: biological properties and clinical application. Clinical Cancer Research, 2007; 13: 4677–4685.
34. Harada T., Miura N. N, Adachi Y., Nakajima M., Yadomae T. and Ohno N. J. Interferon Cytokine Res., 2002; 22: 1227-1239.

35. Xu H, Zou S, Xu X. and Zhang L. Anti-tumor effect of β-glucan from *Lentinus edodes* and the underlying mechanism. Scientific Reports, 2016; 6: 28802. doi:10.1038/srep28802.
36. Yang Z, Xu J, Fu Q, Fu X, and Shu T. Antitumor activity of a polysaccharide from *Pleurotus eryngii* on mice bearing renal cancer. Carbohydr Polym, 2013; 95: 615-620.
37. Yoon, T.J., Kim, T.J., Lee, H., Shin, K.S., Yun, Y.P., Moon, W.K., Kim, D.W. and Lee, K.H. Antitumor metastatic activity of beta-glucan purified from mutated Saccharomyces cerevisiae. International Immunopharmacology, 2008; 8(1): 36-42.
38. Hassan S.S., Al-Saffar A.Z., Ibrahim H.K., Antiangiogenic and immunological effect of β-glucan extracted from *Saccharomyces cerevisiae* HS-1. International Journal of Biotechnology. Photon, 2014; 112: 365-373.