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Evaluation of the Anti-angiogenic Effect of β -glucan Extracted from *P. eryngii*

A Thesis

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Biotechnology.**

By

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I would like to thank my family and my friends for their great support, patience and endurance, I dedicate this work to the soul of my Father who had supported me by every mean in his entire life, I'm proud to see his name completing my name, I also would like to thank my Mother for her great role in my life, without her none of this would have seen the light, I also want to thank my wife and best friend for her endurance and her great love, understanding and support.

Noor Sabah

Summary

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. Samples of dried mushroom *Pleurotus eryngii* were collected from Ministry of Agriculture/Department of Organic Farming in Baghdad. The β -glucan was extracted from the mushroom samples using hot water extraction method. The extracted glucan was confirmed by both High performance liquid chromatography (HPLC) and Fourier transform infrared spectroscopy (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 and protein contents were 250 mg/ml and 0.04 mg/ml respectively.

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 (250, 500, 1000 and 2000 μ g/egg) of β -glucan were used and in comparison with the negative control. Results showed that high doses (500, 1000 and 2000 μ g/egg) 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 dose (250 μ g/egg) showed no significant effect on the neovascularization.

The anti-tumor effect of β -glucan was evaluated in mice which were subcutaneously inoculated with AN3 adenocarcinomas cells line at from thigh toward the shoulder region, different doses (200, 400 and 800 mg/kg/day) of β -glucan were injected intraperitoneally 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.

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List of Abbreviations

Abbreviation	Meaning
HPLC	High Performance Liquid Chromatography
FTIR	Fourier transform infrared spectroscopy
CAM	Chick Chorioallantoic Membrane
IL	Interleukins
VEGF	Vascular endothelial growth factors
DMSO	Dimethyl sulfoxide
ITAM	Immunoreceptor tyrosine based activation motif
TLR	Toll-like receptors
CR	Complement receptor
NF	Nuclear factor
NFAT	Nuclear factor of activated T cells
CARD9	Caspase recruitment domain-containing protein 9
SYK	Spleen tyrosine kinase
ICAM3	Intercellular adhesion molecule 3
MAPK	Mitogen-activated protein kinase
PI3k	Phosphatidylinositol-3 kinase
CD	Cluster of differentiation
Nk	Natural Killers
PDGF	Platelet derived growth factor
FGF	Fibroblast growth factor
EGF	Epidermal growth factor
TGF b	Transforming growth factor
MMPs	Matrix metalloproteinases

TNF	Tumor necrosis factor
BSA	Bovine serum albumin
PBS	Phosphate buffer saline
LNT	Lentinan
SC	<i>S. crispa</i>

Chapter one
Introduction and Literature
Review

1. Introduction and Literature Review

1.1 Introduction

Medicinal mushrooms have been used in traditional Oriental therapies for their antitumor and immunomodulating properties (Liu *et al.*, 1999). Studies have shown that edible mushrooms, which demonstrate medicinal or functional properties, include species of the genera *Lentinus*, *Hericium*, *Grifola*, *Flammulina*, *Pleurotus*, and *Tremella* (Kues and Liu, 2000).

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 (Rosado *et al.*, 2002).

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 (Manzi *et al.*, 1999; Mattila *et al.*, 2000). 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 of them varies according to the source and ranges from 5 to 2000 kDa (Bohn and BeMiller, 1995). These Polysaccharides are believed to be the bioactive ingredients involved in antitumor and anti-inflammatory effects (Wang *et al.*, 1997; Vetvicka *et al.*, 1996).

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 (Wasser, 2002).

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* (Chen and Seviour, 2007).

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 (Pelley and Strickland, 2000; Mayell and Maitake, 2001).

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 (Pelley and Strickland, 2000), inhibitory effects on the growth of tumor cells *in vivo* and affects expression of several important genes in tumor cells (Mantovani *et al.*, 2008). Cell cycle arrest and induction of apoptosis can be seen (Zhang *et al.*, 2006). Activated complement receptors on natural killer cells, neutrophils, and lymphocytes may also be associated with the detected tumor cytotoxicity (Chen and Seviour, 2007), and finally anti-angiogenic properties (Yamamoto *et al.*, 2009).

Angiogenesis is a dynamic process of endothelial proliferation and differentiation. Tumors with high angiogenic activity have been correlated with poor patient survival (Giatromanolaski *et al.*, 1996; Koukouraksi *et al.*, 2000). 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 (Ziche *et al.*, 2004). 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 (Shono *et al.*, 1996). Application of angiogenesis inhibitors is relatively less toxic than conventional chemotherapy and has a lower risk of drug resistance.

Aims of study

According to the information mentioned above, this study aims to assess antiangiogenic and antitumor activity of β -glucan extracted from medicinal mushroom *Pleurotus* spp. This was achieved via the following steps:

- 1- β -glucan extraction.
- 2- Chemical analysis of β -glucan.
- 3-Anti-angiogenic and antitumor assessment of β -glucan.

1.2 Literature Review

1.2.1 Glucans

A glucan molecule is a polysaccharide of D-glucose monomers, linked by glycosidic bonds. Extracellular polysaccharides with various degrees of structural complexity frequently occur in fungal cultures and some of these have been shown to be glucans (Clarke & Stone, 1963; Nordin and Kirkwood, 1965).

According to this structural complexity, glucans can be divided into two groups:

- ❖ α -Glucans, which contains large amounts of glycosidic bonds of the alpha form. Dextran is typical α -glucan which consists of α -1,6 glycosidic linkages between glucose molecules in its straight chain, while branches begin from α -1,3 linkages (and in some cases, α -1,2 and α -1,4 linkages as well) (Purama *et al.*, 2009).
- ❖ β -Glucans, naturally occurring polysaccharides with polybranched β -1,3-(d)-glucans or β -1,6-(d)-glucose side chains, are integral cell wall constituents in a variety of bacteria, yeast, fungi and cereal plant (Babicek *et al.*, 2007; Sener *et al.*, 2007).

Glucan is the major structural polysaccharide of the fungal cell wall, constituting approximately 50–60% of the wall by dry weight (Fleet, 1991; Kapteyn *et al.*, 1999). Polymers of glucan are composed of repeating glucose residues that are assembled into chains through a variety of chemical linkages. In general, between 65% and 90% of the cell wall glucan is found to be β -1,3-

glucan, but other glucans, such as β -1,6, mixed β -1,3 and β -1,4 or α -1,3, and α -1,4 linked glucans, have been found in various fungal cell walls (Bernard and Latge, 2001; Grun *et al.*, 2005). The β -1,3 glucan serves as the main structural constituent to which other cell wall components are covalently attached. As a result, the synthesis of β -1,3-glucan is required for proper cell wall formation and the normal development of fungi.

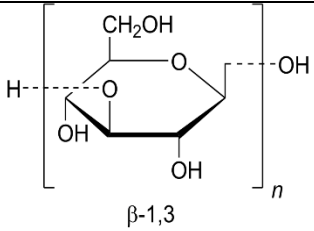
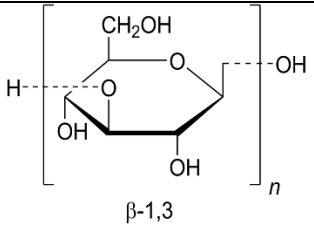
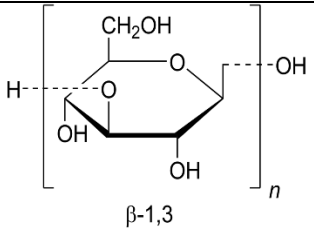
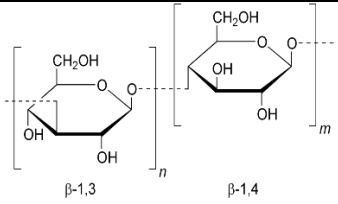
Polymers of β -1,3 glucan, like those of chitin, are generated by enzyme complexes associated with the plasma membrane and extruded into the extracellular space by means of vectorial synthesis (Douglas, 2001; Manners *et al.*, 1973). As with chitin, this mode of synthesis promotes the association of nascent glucan chains within the cell wall space and facilitates their integration into the cell wall. This integration occurs at points of active cell wall synthesis, and the glucan synthase complexes, similar to those generating chitin, are primarily localized to areas of cell growth and budding or branching (Delly and hall, 1999; Beauvais *et al.*, 2000). Glucan synthase catalyzes the formation of long linear chains of glucan, each composed of approximately 1,500 glucose residues connected via beta-1,3-linkages. Within each long glucan chain, the carbon-6 positions of approximately 40–50 glucose residues become sites at which additional β -1,3-glucans are attached to generate a branched structure (Fontaine *et al.*, 2000). The branched glucans can then be cross-linked together and to chitin and mannoproteins to provide the cell wall with mechanical strength and integrity (Kollar *et al.*, 1997).

1.2.2 Chemistry of β -glucans

Glucans are arranged in six-sided D-glucose rings connected linearly at varying carbon positions depending on the source, although most commonly β -glucans include a 1-3 glycosidic link in their backbone (Zekovic and Djordje, 2008). Although technically β -glucans are chains of D-glucose polysaccharides

linked by β -type glycosidic bonds, by convention not all β -D-glucose polysaccharides are categorized as β -glucans. Differences in molecular weight, shape, and structure of β -glucans dictate the differences in biological activity (Table 1-1) (Chu and Yifang, 2014).

Table (1-1): β -glucan Structures

Source	Backbone	Branching	Solubility in water	Reference
Bacteria	 β -1,3	None	Insoluble	(Mcintosh, 2004)
Fungus	 β -1,3	Short β -1,6 branching	Insoluble	(Han and Deuk, 2008)
Yeast	 β -1,3	Long β -1,6 branching	Insoluble	(Volman and Julia, 2007)
Cereal	 β -1,3 α -1,4	None	Soluble	(Chu and Yifang, 2014)

Cellulose is not typically considered a β -glucan, as it is insoluble and does not exhibit the same physicochemical properties as other cereal or yeast β -glucans. Some β -glucan molecules have branching glucose side-chains attached

to other positions on the main D-glucose chain, which branch off the β -glucan backbone. In addition, these side-chains can be attached to other types of molecules, like proteins, as in Polysaccharide-K (Sikora and Per, 2012). The most common forms of β -glucans are those comprising D-glucose units with β -1,3 links. Yeast and fungal β -glucans contain 1-6 side branches, while cereal β -glucans contain both β -1,3 and β -1,4 backbone bonds (Chu and Yifang, 2014). The frequency, location, and length of the side-chains may play a role in immunomodulation.

1.2.3 Types of β -Glucan

- **Linear (1-3)- β -Glucans (Curdlan)**

Curdlan, recognized as a (1-3)- β -glucan by its staining with either the Aniline blue dye or fluorochrome, is found as capsular polysaccharide in Gram-negative bacteria belonging to the rhizobiaceae (e.g. *Agrobacterium* and *Rhizobium*) and the Gram positive *Cellulomonas falvigena* (Buller and Voepel, 1990; Kenyon and Buller, 2002; Kenyon *et al.*, 2005) and a *Bacillus* sp. (Gummadi and Kumar, 2005). Curdlan is a linear, unbranched (1,3)- β -glucan as shown in (Fig. 1-1) (Harada *et al.*, 1968; Nakanishi *et al.*, 1976) which may have as many as 12000 Glucose units (Futatsuyama *et al.*, 1999). Curdlan is insoluble in water, alcohols and most organic solvents but dissolves in dilute bases (0.25 M NaOH) and DMSO.

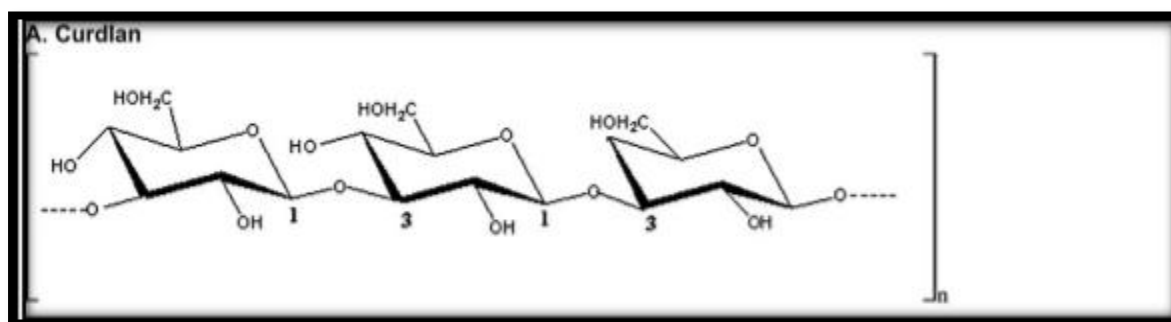


Figure (1-1): Chemical structure of linear β -(1,3)-D-Glucans (Laroch and Michaud, 2007)

- **Cyclic (1,3;1,6)- β -Glucans**

Water-soluble cyclic (1,3;1,6)- β -glucans are produced by the *Legume* symbionts, *Bradyrhizobium japonicum*, *Rhizobium loti*, *Azorhizobium caulinodans*. *Bradyrhizobium japonicum* strains, growing as free-living cultures or as bacterioids, synthesize a mixture of cyclic (1,3;1,6)- β -glucans that are neutral, unsubstituted and have ring sizes of 10-13 units (Fig. 1-2) (Miller *et al.*, 1990; Rolin *et al.*, 1992; Gore and Miller, 1993; Inon de Iannino and Ugalde, 1993).

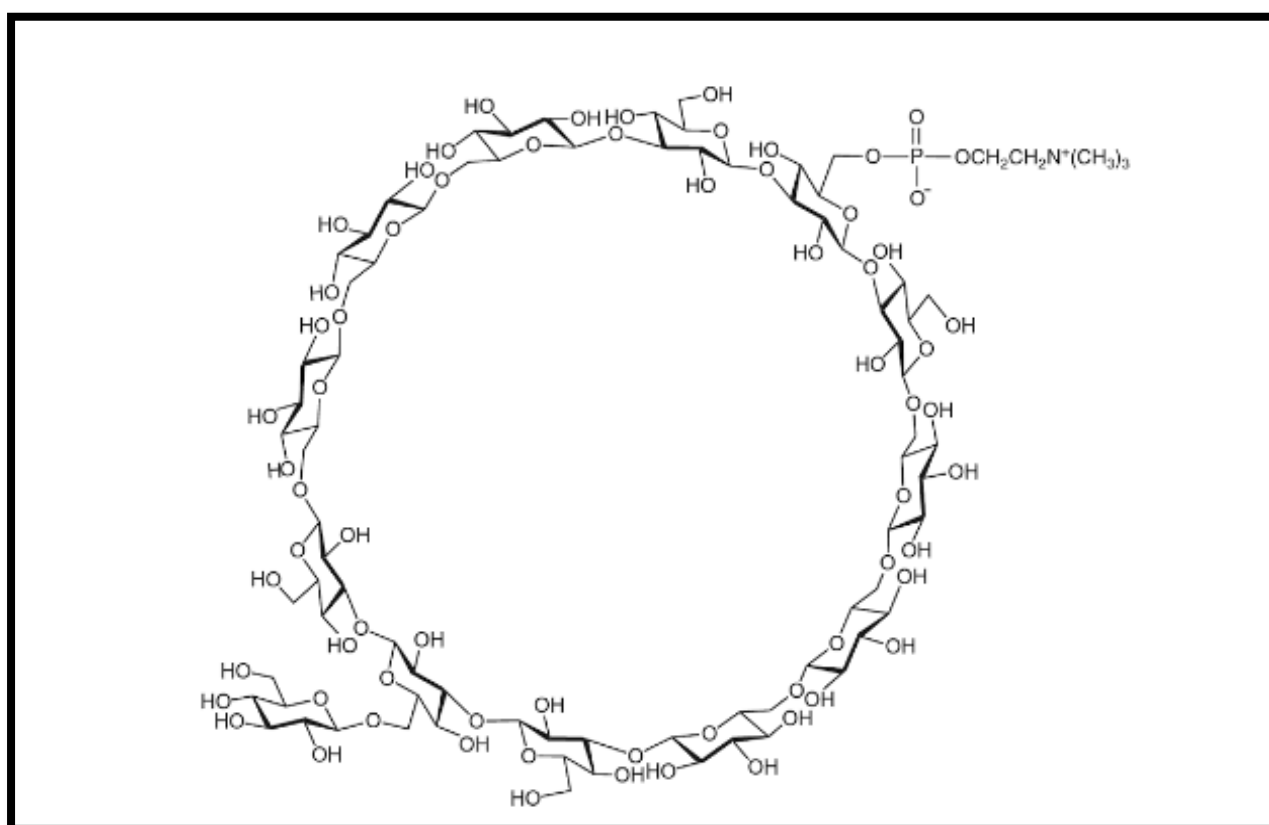


Figure (1-2): Schematic representation of the structure of cyclic (1,3;1,6)- β -D-glucan (Rolin *et al.*, 1992)

- **β -(1,3) (1,6)-D-Glucans**

It is an important component of the cell wall of the yeast and fungi that maintain the structural function for the permeability of the cell wall (Lee *et al*, 2001). This type of glucans contained chains of glucose unit linked by β -(3-1) bond that known as β (1-3)-D-Glucosyl which is a backbone to the compound that branched by unit of glucose linked by β (1-6) chain that known as β (1-6)-D-Glucosyl. This type of glucan is characterized by a wide variety in the type of branch and number of the glucose unit, so they may be linked irregular or regular in the chain (Sutherland, 2001). The β glucan is produced from different organisms (Table 1-2) and *Pleurotus* spp. are among the important sources of glucan

Table (1-2): Sources of glucans

Structure	Trivial name	Source	Reference
(1→3)- β -d-glucan	Callose	Higher plants	(Chen and Kim, 2009)
(1→6)- β -d-glucan	Pustulan	<i>Lasallia pustulata</i>	(Pereyra <i>et al</i> , 2003)
β -(1→3)branched by(1→6)- β -d-glucan	Lentinan	<i>Lentinula edodes</i>	(Zhang <i>et al.</i> , 2011)
(1→4)(1→6)- α -d-glucan	Pullulan	<i>Aureobasidium pullulans</i>	(Singh <i>et al.</i> , 2008)
(1→3)- β -d-glucan	Paramylon	<i>Euglena gracilis</i>	(Kiss <i>et al.</i> , 1987)
β -(1→3)branched by(1→6)- β -d-glucan	Cell walls	<i>Pleurotus eryngii</i> , <i>Pleurotus ostreatus</i> ,	(Carbonero <i>et al.</i> , 2006)
(1→3)- β -d-glucan	Curdlan	<i>Agrobacterium</i> sp.	(Marchessault and Deslandes, 1979)
(1→3)(1→4)- β -d-glucan	Cereal β -d-glucan	Higher plants(Cereals)	Lui and White, 2011)

(1→3)(1→4)-β-d-glucan	Calocyban	<i>Calocybe indica</i>	(Mandal <i>et al.</i> , 2010)
(1→3)(1→6)-β-d-glucan	Grifolan	<i>Grifola frondosa</i>	(Tada <i>et al.</i> , 2009)

1.3 *Pleurotus* spp.

Since centuries, mushrooms have been used as nourishment throughout the world due to its rich nutrients such as proteins, carbohydrates, lipids, minerals (phosphorous and potassium), and vitamins (Smith *et al.*, 2002). Mushroom nowadays considered as one of the most important functional food with many well-known therapeutic applications (El-Enshasy and Hatti-Kaul, 2013; Ozturk *et al.*, 2015).

Pleurotus is a genus of gilled mushrooms which includes one of the most widely eaten mushrooms, *P. ostreatus* called oyster, abalone, or tree mushrooms, and are some of the most commonly cultivated edible mushrooms in the world (Chang *et al.*, 2004). The genus *Pleurotus* (oyster mushroom) is an organoleptic fast growing fungus, which belongs to basidiomycota group, about seventy species are discovered for this genus (Kong, 2004; Gargano *et al.*, 2013).

Pleurotus. eryngii is the largest species in the oyster mushroom genus, *Pleurotus*, which also contains the oyster mushroom *Pleurotus ostreatus*. It has a thick, meaty white stem and a small tan cap (in young specimens). Its natural range extends from the Atlantic Ocean through the Mediterranean Basin and Central Europe into Western Asia and India (Fig. 1-3) (Zervakis *et al.*, 2001).

Pleurotus eryngii basidiomata have a high nutritional value, they are especially rich in carbohydrates (9.6% of fresh weight), and a significant

amount corresponds to dietary fibers (4.64% of fresh weight; 4.11% is insoluble and 0.53% is soluble dietary fibers), chitin (0.50% of fresh weight), and polysaccharides (0.41% of fresh weight). The total nitrogen content is around 5.30%, and protein content is between 1.88% and 2.65%. The most abundant amino acids are aspartic acid, glutamic acid, and arginine. Significant concentrations of vitamins (C, A, B₂, B₁, D, and niacin) and minerals (especially K, Mg, Na, and Ca), very low amounts of lipid (0.8% of fresh weight), and high moisture (between 86.6% and 91.7%) are present in the basidiomata (Manzi *et al.* 1999; 2004).



Figure (1-3): *Pleurotus eryngii* (Zervakis *et al.*, 2001)

Pleurotus eryngii is reported to synthesize various biologically active compounds. Polysaccharides (ex. β -1,3-glucans), among others, are the main biologically active compounds in *P. eryngii*, which are classified pharmacologically as biological response modifiers, due to the enhancement of the host immune system as a response to various diseases. Besides polysaccharides, numerous other biologically active compounds were isolated

from *P. eryngii* basidiomata. Lovastatin has been found to be an effective natural compound that can decrease cholesterol level in blood. The studies reported involving different methods and compounds derived from *P. eryngii* have led to the general belief that this fungus has anti-hypertensive, antioxidant, anti-hypercholesterolic, anti-hyperglycemic, immunomodulating, antitumor, antibacterial, antiviral, antifungal, antiinflammatory, and anti-osteoporotic effects (Wasser and Weis, 1999).

In recent years, various methods have been developed to extract the anticancer polysaccharides (β glucan) from mushroom fruit-bodies, mycelium and liquid media (Mizuno, 1999).

Many studies demonstrated that the extracted β - glucan is either soluble or particulate. The solubility of β -glucans is associated with the degree of polymerization β -glucans can be classified according to their solubility properties into (Mario *et al.*, 2007):

- Alkali insoluble, acetic acid insoluble (1,3)- β -glucan.
- Alkali-soluble (1,3)- β -glucan.
- Highly branched (1,3),(1,6)- β -glucan.

1.4 The biological activity of β -glucan

1.4.1 The effects of β -glucans on the immune system

Current data suggests that β -glucans are potent immunomodulators with effects on both innate and adaptive immunity (Fig.1-4).

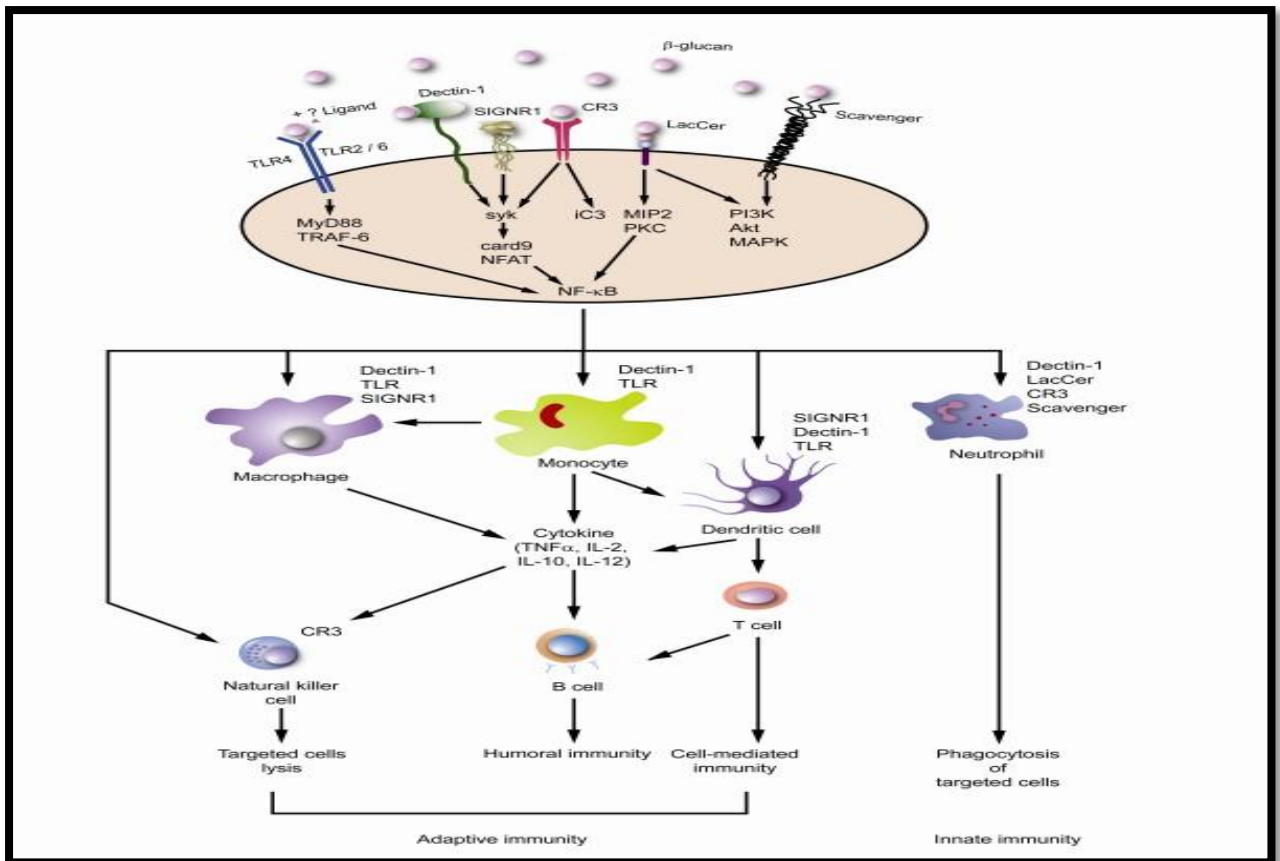


Figure (1-4): Immune activation induced by β -glucans (Schorey and Lawrence, 2008)

The ability of the innate immune system to quickly recognize and respond to an invading pathogen is essential for controlling infection. Dectin-1, which is a type II transmembrane protein receptor that binds β -1,3 and β -1,6 glucans, can initiate and regulate the innate immune response (Sun and Zhao, 2007; Herre *et al.*, 2004). It recognizes β -glucans found in the bacterial or fungal cell wall with the advantage that β -glucans are absent in human cells. It then triggers effective immune responses including phagocytosis and proinflammatory factors production, leading to the elimination of infectious agents (Schorey and Lawrence, 2008; Brown, 2006). Dectin-1 is expressed on cells responsible for innate immune response and has been found in macrophages, neutrophils, and dendritic cells (Taylor *et al.*, 2002). The Dectin-1 cytoplasmic tail contains an immunoreceptor tyrosine based activation motif (ITAM) that signals through

the tyrosine kinase in collaboration with Toll-like receptors 2 and 6 (TLR-2/6) (Schorey and Lawrence, 2008; Ganter *et al.*, 2003; Herre *et al.*, 2004). The entire signaling pathway downstream to dectin-1 activation has not yet been fully mapped out but several signaling molecules have been reported to be involved. They are NF- κ B (through Syk-mediate pathway), signaling adaptor protein CARD9 and nuclear factor of activated T cells (NFAT) (Goodridge *et al.*, 2007; Rogers *et al.*, 2005). This will eventually lead to the release of cytokines including interleukin IL-12, IL-6, tumor necrosis factor TNF- α , and IL-10. Some of these cytokines may play important role in the cancer therapy. On the other hand, the dendritic cell-specific ICAM-3-grabbing non-integrin homolog, SIGN-related 1 (SIGNR1) is another major mannose receptor on macrophages that cooperates with the Dectin-1 in non-opsonic recognition of β -glucans for phagocytosis (Taylor *et al.*, 2004). Furthermore, it was found that blocking of TLR-4 can inhibit the production of IL-12 p40 and IL-10 induced by purified Ganoderma glucans (PS-G), suggesting a vital role of TLR-4 signaling in glucan induced dendritic cells maturation. Such effect is also operated via the augmentation of the I κ B kinase, NF- κ B activity and MAPK phosphorylation. One additional point to note is that those studies implied the interaction between β -glucans and TLR all used non-purified β -glucans, therefore the actual involvement of pure β -glucans and TLR remains to be proven (Lin *et al.*, 2005).

Other possible receptors and signaling pathways induced by β -glucans are less definite at the moment. For example, lentinan, a form of mushroom derived β -glucans, has been found to bind to scavenger receptor found on the surface of myeloid cells and triggers phosphatidylinositol-3 kinase (PI3K), Akt kinase and p38 mitogen-activated protein kinase (MAPK) signaling pathway (Rice *et al.*, 2002). *Candida albicans* derived β -glucans but not other forms of pathogenic fungal β -glucans can bind to Lac Cer receptor and activate the PI-3K pathway

in controlling the neutrophil migration (Sato *et al.*, 2006), but such activation pathway may involve other molecules found in the *Candida* derived β -glucans.

Another mechanism of β -glucan action is mediated via the activated complement receptor 3 (CR3, also known as CD11b/CD18), which is found on natural killer (NK) cells, neutrophils, and lymphocytes. This pathway is responsible for opsonic recognition of β -glucans leading to phagocytosis and reactor cells lysis. β -glucans bind to the lectin domain of CR3 and prime it for binding to inactivated complement 3b (iC3b) on the surface of reactor cells. The reactor cells can be of any cell type including cancer cells tagged with monoclonal antibody and coated with iC3b. The β -glucans-activated circulating cells such as the CR3 containing neutrophils will then trigger cell lysis on iC3b-coated tumor cells (Hong *et al.*, 2004). Similarly, majority of the human NK cells express CR3 and it was shown that opsonization of NK cells coated with iC3b leads to an increase in the lysis of the target. The beta chain of the CR3 molecule (CD18) rather than the alpha chain (CD11b) is responsible to the β -glucan binding (Klein *et al.*, 1990; Di Renzo *et al.*, 1991).

1.4.2 Anti-tumor effects of β -glucans

Cancer is defined as an uncontrolled cell proliferation induced by many factors including environmental chemicals, viruses, bacteria, hormones, and chronic inflammation (Nam *et al.*, 2005). There are three developmental stages recognized. The first is called initiation, in which a mutagen binds to the cell DNA and causes damage, which by itself is usually insufficient to induce tumor production. The second stage is called activation of a tumor promoter that leads to the formation of small benign tumors. In the third stage is progression, the normal tight control over the cell cycle is lost, which results in uncontrolled cell proliferation (Borchers *et al.*, 2004; Nam *et al.*, 2005). Cancer is a worldwide disease which is causing serious damage to human health, how to conquer cancer is one of the most important research topics on the medicine. Recently,

some natural active component has been discovered and purified from *Pleurotus eryngii*, including polysaccharide, polysaccharide-protein, diterpenoid, triterpene. These components exhibited the significant anti-cancer activity (Yang *et al.*, 2013).

Biological studies have demonstrated that polysaccharides from *Pleurotus eryngii* played a main role in the enhancement of the antitumor activity. exopolysaccharides from *Pleurotus eryngii* exhibited higher antitumor ability of human hepatoma cells *in vitro*. The antitumor activity increased in a concentration dependent manner and reached to 61.4% at the concentration of 400 µg/ml (Jing *et al.*, 2013).

It was found that fungal β -glucans appear to beneficially influence both cancer promotion and progression (Takaku *et al.*, 2001; Nilsson *et al.*, 2004; Nam *et al.*, 2005), and such treatment of rats has led to formation of much smaller tumors than those seen in controls (Vetvicka and Yvin, 2004). Fungal β -glucans also have synergistic effects with monoclonal antibodies used in cancer treatment. Monoclonal antibody therapy targets key components of the biological pathways involved in carcinogenesis. Furthermore, yeast β -glucans given orally with monoclonal antibody therapy increased neuroblastoma tumor regression and long-term survival in mice (Cheung *et al.*, 2002; Cheung and Modak, 2002; Yan *et al.*, 2005).

1.4.3 Anti-Angiogenic Effect of β Glucan

It has been proposed that anti-angiogenesis may be another pathway that β -glucans exert the anti-cancer property. Yamamoto *et al.* (2009) investigated the effect of oral administration of *Sparassis crispa* (SC, or known as Hanabiratake in Japan) on angiogenesis. SC is an edible mushroom with medicinal properties that contains more than 40% β --glucan. they found that SC can suppress the growth of the primary tumour and also the extent of the metastatic foci in the

lung, correlating with the inhibition of tumor induced-angiogenesis. Similarly, using *Coriolus versicolor* mycelia, it was found that there was a reduction of tumor weight and vascular density via suppression of vascular endothelial cell growth factor (VEGF) gene expression (Ho *et al.*, 2004). Anti-angiogenesis effects induced by β -glucans may be acting through an indirect manner and the underlying mechanisms are an emerging area for future studies.

The VEGF is the key of the angiogenesis, some studies reported that fugal polysaccharide from different fungi are commercially available and have inhibitory effects on tumor angiogenesis and tumor growth in mice (Chen *et al.*, 2005).

1.4.3.1 Angiogenesis

Angiogenesis is a complex process in which there is growth of new blood vessels from the pre-existing ones and is an essential phenomenon for the growth and survival of solid neoplasms (Fig.1-5) (Hasina and Lingin, 2001).

Tumor angiogenesis is the proliferation of blood vessels penetrating the cancerous growth for the supply of nutrients and oxygen (Grupta and Qin, 2003). Angiogenesis is a requisite not only for continued tumor growth, but also for metastasis (Kumar *et al.*, 2004). Because adequate vascular response is critical for the initial development as well as the continued growth of solid tumors, much attention is focused on the use of angiogenesis inhibitors as an adjunct to other forms of therapy for preventing development of malignant neoplasms (Hori *et al.*, 1991).

Folkman (1995) demonstrated that solid tumors cannot grow larger than 2-3 mm diameter without inducing their own blood supply. Tumor angiogenesis starts with the release of molecules by tumor cells that send signals to the surrounding normal host tissue, activates certain genes to make protein that encourage growth of new blood vessels (Grupta and Qin, 2003).

Antiangiogenesis is inhibition of blood vessel growth, It's being investigated as a way to prevent the growth of tumor and other angiogenesis-dependent diseases. Pharmacological inhibition interferes with the angiogenic cascade or the immature neovasculature with synthetic or semi-synthetic substances, endogenous inhibitors or biological antagonists (Ribatti, 2010).

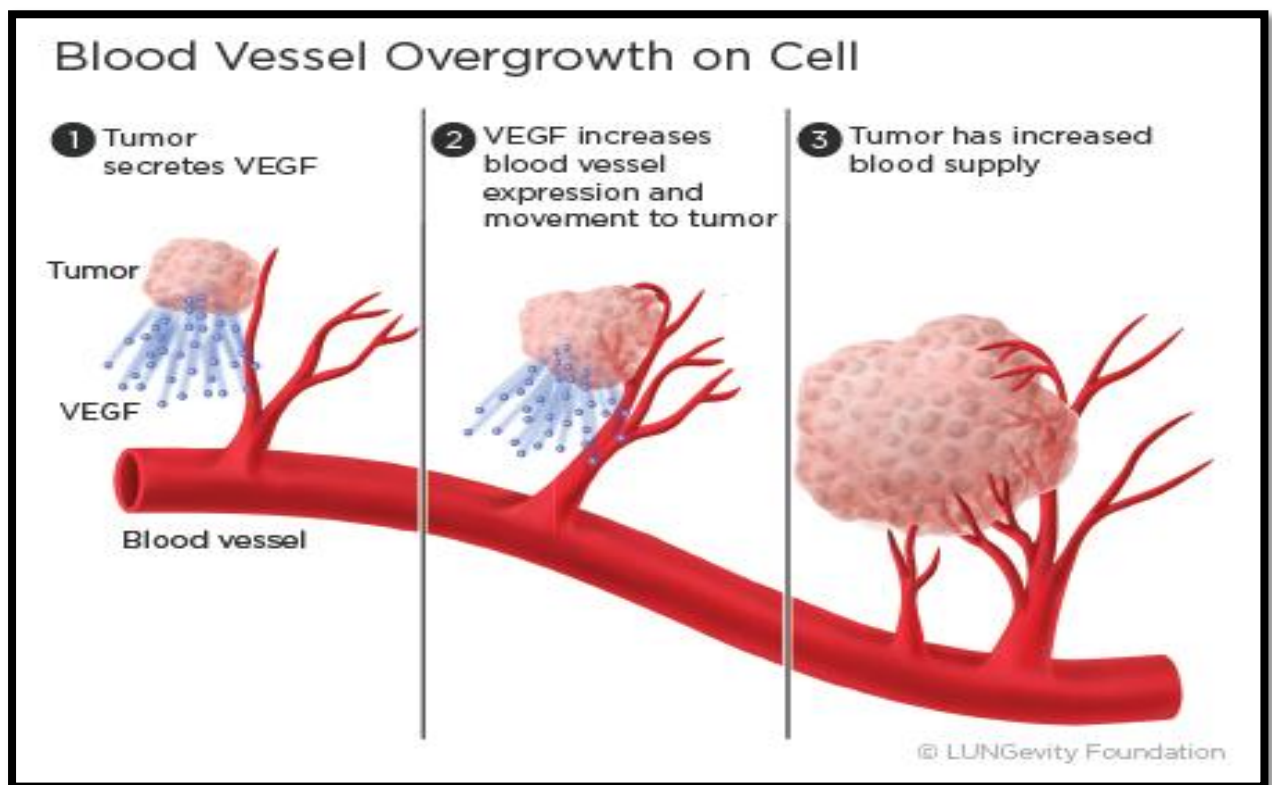


Figure (1-5): The process of angiogenesis (Hasina and Lingen, 2011)

1.4.3.2 Stimulators of Angiogenesis

Most blood vessels in an adult organism remain quiescent but have the capability to divide in response to stimulus and result in angiogenic process. The molecules that are the positive regulators of angiogenesis are:

A-VEGF (Vascular endothelial growth factor): VEGF also known as vascular permeability factor (VPF) is a heparin binding protein and its level is increased in various tumors (Kohn *et al.*, 1992).

Actions of VEGF include:

- Powerful inducer of angiogenesis (Leung *et al.*, 1989).
- Stimulates growth and proliferation of endothelial cells (Ferrara and Davis Smyth, 1997).
- Act as survival factor for endothelial cells (Gerber *et al.*, 1998).
- Prevent the apoptosis of endothelial cells (Gerber *et al.*, 1998).
- Regulates the vascular permeability (Senger *et al.*, 1983).

B- Platelet Derived Growth Factor (PDGF): Family of PDGF ligand is composed of four structurally related soluble peptides in the form of five different homodimers and heterodimers and involved in vessel maturation and recruitment of pericytes (Karamysheva, 2008).

C- Fibroblast Growth Factor (FGF): It is comprised of 23 different proteins and classified into six different groups. These ligands are among the earliest angiogenic factors and involved in promoting cell proliferation, migration and differentiation of vascular Endothelial cells (Grupta and Qin, 2003).

D-Epidermal Growth Factor (EGF): It is comprised of 11 members and four EGF receptors. Activation of Epidermal growth factor receptor pathway results

in up regulation of proangiogenic factors such as VEGF and thus viewed as indirect regulator of angiogenesis (Carla *et al.*, 2012).

E- Transforming Growth Factor- β (TGF β): TGF β is produced by nearly every cell type and participates in angiogenesis, embryonic development and wound healing and has potent growth inhibition properties. It has both pro and anti-angiogenic properties, depending on its levels. Low levels promote angiogenesis by up regulating angiogenic factors and proteases and high levels inhibit endothelial cells growth and proliferation by preventing phosphorylation of pRB and thereby arresting endothelial cells at late G1 phase (Grupta and Qin, 2003).

F-Matrix Metalloproteinases (MMPs): MMPs induce tumor angiogenesis by degrading extracellular matrix and releasing angiogenic mitogens that are stored in the matrix. MMP-9 and MMP-2 proteolytically cleave and activate latent TGF- β and promote tumour angiogenesis (Grupta and Qin, 2003).

G-Angiopoietins: The angiopoietins, Ang1 and Ang2, are required for the formation of mature blood vessels, as demonstrated by mouse studies (Gavin, 2004).

1.4.3.3 Angiogenesis Inhibitors

Because an adequate vascular response is essential for the initial development and the continued growth of the solid tumors so now a day's anti angiogenic therapy is an attractive modality for preventing the development of malignant neoplasm (Kim *et al.*, 1993). The angiogenic inhibitors can be either endogenous (present within the body) or synthetic (drugs). The various Endogenous Angiogenesis inhibitors are as follows:

A. Interferon

Interferon is the members of secreted glycoproteins which directly or indirectly inhibit the tumor angiogenesis and growth. Administration of optimal dose of IFN α/β decrease the expression of β - FGF mRNA and protein, microvessel density and also induces the apoptosis of endothelial cells (Slaton *et al.*, 1999). IFN- γ induces its antiangiogenic effects through the secretion of IFN γ – inducible protein 10 (IP-10) and monokine (Jonasch and Haluska, 2001).

B. Interleukins

The structure of interleukins (ILs) determines its function to play a role in either promoting or inhibiting angiogenesis, IL- 12 suppresses the expression of VEGF mRNA, promotes the apoptosis and inhibits proliferation rate in human tumors and reduce tumor vessel density (Oshikawa *et al.*, 2010; Duda *et al.*, 2000). IL-10 down regulate the synthesis of VEGF, IL-1 β , TNF α , IL-6, MMP 9 in tumor associated macrophages (Huang *et al.*, 1991).

C. Angiostatin

It is a 38 KDa internal fragment of plasminogen and its antiangiogenic effect is due to down regulation of VEGF expression within tumors (Hajitou *et al.*, 2002). Binding of angiostatin to plasma membrane localized ATP synthetase suppress the endothelial –surface ATP metabolism and thus down regulate the EC (endothelial cell) proliferation and migration (Moster *et al.*, 1999). According to many studies angiostatin treatment significantly increases the apoptosis of EC (Griscelli *et al.*, 1998).

D. Endostatin: It is a 20 KDa fragment of type XVIII collagen and inhibits ECs proliferation, angiogenesis and tumor growth (Kim *et al.*, 2002).

1.5 Studying Anti-Angiogenesis by Chick Embryo Chorioallantoic Membrane Assay (CAM Assay)

The classical assays for studying angiogenesis *in vivo* include the rabbit ear chamber, the mouse dorsal skin and air sac, the chick embryo chorioallantoic membrane (CAM) (Norrby, 2006). Angiogenesis assays *in vitro* have allowed important progress in elucidating the mechanism of action of several angiogenic factors and inhibitors. The main determinants dictating the choice of method are their cost, ease of use, reproducibility, and reliability. However, *in vivo* angiogenesis assays may be very sensitive to environmental factors and not readily accessible to biochemical analysis. Also, their interpretation is frequently complicated by the fact that the experimental condition adopted may inadvertently favor inflammation (Ribatti, 2010).

The CAM can be also cultured either *in vivo* or *ex-vivo* as a shellless culture in Petri dishes and plastic wrap/cup apparatus. There is no clear evidence that there is any significant difference between data derived using or shell-less culture method. It has been demonstrated that survival rate of eggs cultured *ex vivo* is the major success limiting step in this culture technique (Hassan *et al.* 2014).

Chapter Two

Materials and Methods

2. Materials and methods

2.1 Materials

2.1.1 Equipment and Apparatuses

The equipment and apparatus used throughout this study are mentioned as follows:

Equipment	Company	Origin
Autoclave	Express	Germany
Balance	Ohans	France
Centrifuge	Hermle Z200A	Germany
Compound Light Microscope	Olympus	Japan
Cooling Centrifuge	Harrier	UK
High Speed Centrifuge	Supra 22K	Korea
HPLC, FT-IR	Shimadzu	Japan
Incubator	Termaks	UK
Laminar air flow hood	Heraeus	Germany
Micropipette	Witey	Germany
Oven	Memmert	Germany
pH meter	Milwaukee	USA
Sensitive Balance	Kern	Germany
Shaker Incubator, Water bath, Distillator	GFL	Germany
Spectrophotometer	Aurora Instrument Ltd	England
Vortex	Giffin	England

2.1.2 Chemicals

Chemicals	Company	Origin
Coomassie Brilliant Blue G-250	LKB	Sweden
Chloroform	BDH	England
Ethanol Absolute	BDH	England
Eosin	BDH	England
Glucose	BDH	England
Hematoxylin	BDH	England
Phenol	Sigma	Germany
Phosphoric Acid	BDH	England
Tris-HCl	Fluka	Germany

2.1.3 Standards

Standard	Company	Origin
β -1,3-glucan from <i>Euglena gracilis</i>	Sigma	Germany

2.1.4 Solutions, Reagents and Stains

2.1.4.1 Solutions for Carbohydrate Determination (Dubois *et al.*, 1956)

A. Glucose Stock Solution

It was prepared by dissolving 1mg of glucose in 10ml distilled water to obtain 100µg/ml.

B. Phenol Solution (5%)

Phenol (5g) was dissolved in 100ml distilled water.

C. H₂SO₄ Solution (98%).

2.1.4.2 Solutions for Protein Estimation (Bradford, 1976)

A. Coomassie Brilliant Blue G-250 Stain

It was prepared by dissolving 0.1g of Coomassie Brilliant Blue-G250 in 50ml of 95% ethanol, then 100ml of 85% phosphoric acid was added with agitation and the volume was completed to one liter with distilled water. The mixture was filtrated via Wattman filter paper (No.1) and kept in a dark bottle.

B. Tris-HCl Buffer

It was prepared by dissolving 0.3g of Tris-HCl in 100ml distilled water, pH was adjusted to 7.5.

C. Bovine Serum Albumin (BSA)

It was prepared by dissolving 10mg of BSA in 10ml of Tris-HCl buffer.

2.1.4.3 Solutions Used for Glucan Extration (Wood, 1993)

A. HCl (2M)

It was prepared by mixing 7.5ml HCl with 100ml distilled water.

B. Na₂CO₃ (20%)

It was prepared by dissolving 20g of Na₂CO₃ in 100ml of distilled water.

2.1.4.4 Solutions Used in Histological Study of Mice**A. Hematoxylin Stain**

The stain solution was ready supplied by the laboratories of Baghdad University/College of Medicine. It was used to stain the histological sections of mice.

B. Eosin Stain

The stain solution was ready supplied by the laboratories of Baghdad University/College of Medicine. It was used to stain the histological sections of mice.

C. Formalin (10%)

It was prepared by mixing 10ml of formalin with 90ml of distilled water.

2.1.5 Tumor Cell line (Al-Shammari *et al.*, 2008)

Ahmed Majeed 2003 (AM3) also named (AN3) mammary adenocarcinoma transplantable tumor line was supplied from ICCMGR, Experimental Therapy Department/Al-Mustansiriya University.

2.2 Methods**2.2.1 Sample collection of Mushroom**

Samples of dried mushroom *Pleurotus eryngii* were supplied from Ministry of Agriculture/Department of Organic Farming in Baghdad.

2.2.2 Identification of *Pleurotus* spp.

The mushroom samples were identified by Dr. Ahmed Kareem\Senior of Agronomist at the Ministry of Agriculture/Department of Organic Farming.

2.2.3 β -Glucan Extraction from *Pleurotus eryngii*

β - glucan was extracted using hot water extraction method with simple manipulation (Wood, 1993):

- 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 proteins and centrifuged 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.
- The pellet was homogenized with ethanol absolute and then dried by oven at 60°C.

2.2.4 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 μ g/ml standard, And then 20 μ l 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.

2.2.5 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.

2.2.6 Determination of Carbohydrate Concentration

Carbohydrate concentration was determined by phenol - H₂SO₄ method originally described by Dubois et al., (1956) as follows:

- Different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml) were prepared from glucose stock solution (2.1.5.1) with final volume of 1 ml. Then 1 ml of phenol solution (5%) was added to each tube with mixing.
- A volume of 5 ml from H₂SO₄ was added to the mixture with vigorous mixing, and then left to cool at room temperature.
- The absorbance at 490 nm was measured; the blank was prepared from 1 ml of distilled water, 1 ml phenol solution (5%) and 5 ml of H₂SO₄.
- A standard curve was plotted between the glucose concentrations against the corresponding absorbance (Figure 2-1).
- The carbohydrate concentration of glucan was estimated by taking 1ml of 1 mg/ml glucan solution and subjected to the same previous addition and read the absorbance at 490nm. The carbohydrate concentration was calculated from the standard curve (Figure 2-1).

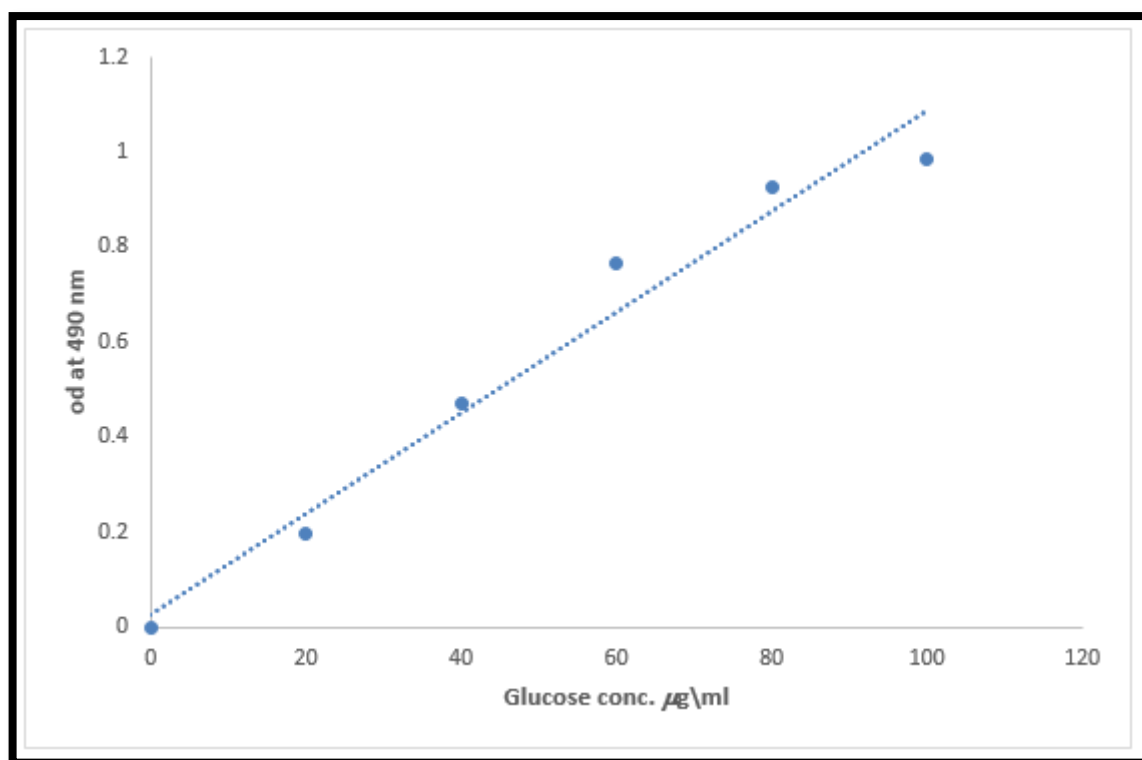


Figure (2-1): Standard curve of glucose

2.2.7 Estimation of Protein Concentration

Protein concentration was determined according to Bradford, (1976) as follow: A standard curve of bovine serum albumin was carried out by preparation different concentrations from BSA stock solution according to the following:

BSA (μl) Stock Solution	Tris-HCl Buffer (μl)	Protein Concentration (μl/ml)	Final Volume (ml)
20	80	20	0.1
40	60	40	0.1
60	40	60	0.1
80	20	80	0.1
100	0	100	0.1

- A volume of 2.5 ml of Coomassie Brilliant Blue G-250 dye was added to each concentration mixed and left to stand for 2 min at room temperature.
- The absorbance at 595 nm was measured; the blank was prepared from 0.1 ml of Tris-HCl buffer and 2.5 ml of the dye reagent.
- A standard curve was plotted between the BSA concentrations against the corresponding absorbance of the bovine serum albumin.
- Glucan sample (0.1ml of 100 μ g/ml Tris-HCl) was treated as the same previous steps. The protein concentration was calculated from the standard curve (Fig.2-2).

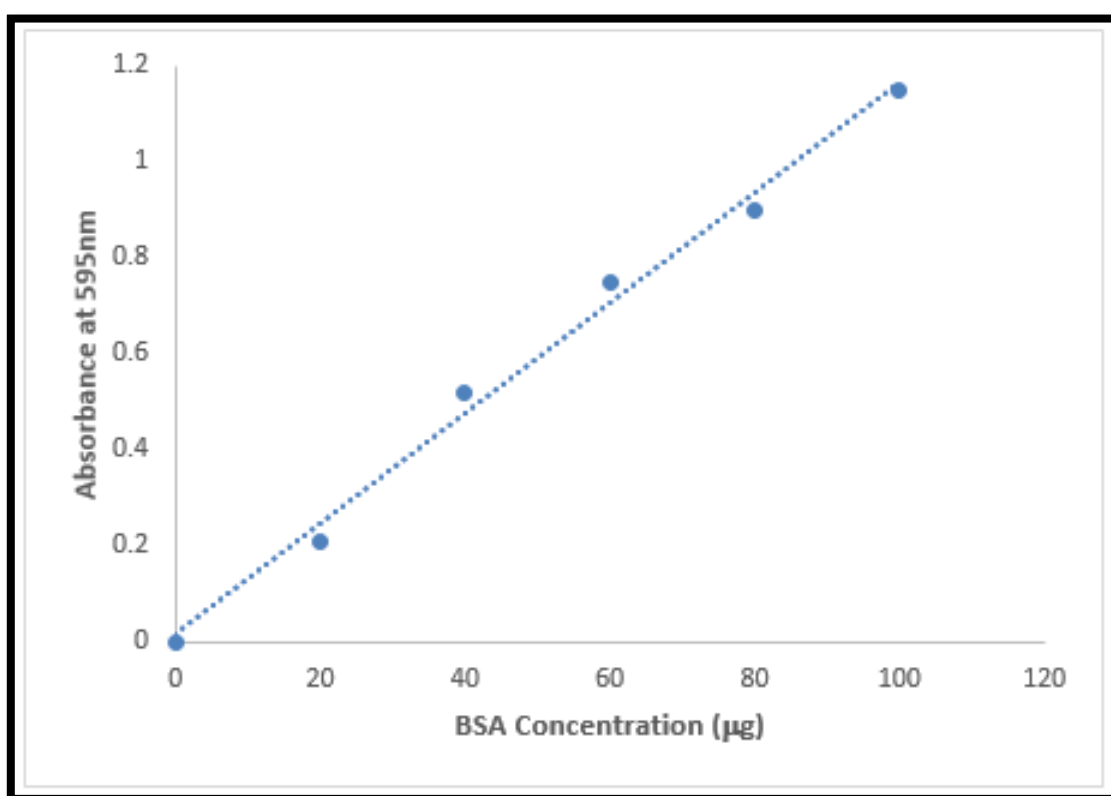


Figure (2-2): Standard Curve of BSA

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

Ex vivo anti-angiogenic activity of glucan was measured by CAM assay as described by (Li *et al.*, 2004). A group of 25 fertilized eggs about 7-10 days-old were incubated at 37.5 °C with 55% relative humidity. On the eighth day, a 1cm² window was carefully created on the broad side of each egg shell, which can candle the egg to assure existence of embryonic blood vessels. A volume of glucan (20 μ l) containing (250, 500, 1000 and 2000 μ g/egg) 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 of each egg, 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.

2.2.9 Tumor Induction and Treatment Procedures

2.2.9.1 Tumor Induction

According to the procedure described by Al-Shammari *et al.*, (2008). 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 (2.1.5) 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. The following protocol was followed to perform the transplantation process (Al-Shammari *et al.*, 2011), 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 withdrawn 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 left. 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 was transplanted through insertion of a needle (gage No.18) subcutaneously from thigh region toward the shoulder region where the injection was performed.



Figure (2-3): Mice bearing AN3 mammary adenocarcinoma cell Line.

2.2.9.2 Tumor Treatment with β -Glucan

This experiment was conducted to evaluate the effect an anti-tumor activity of β glucan. The experimental mice were divided into four groups, three groups of mice were intraperitoneally injected with β -glucan suspended in physiological saline at a dose of (200, 400 and 800 mg/kg/day) for 10 consecutive days while the vehicle-control mice received an equivalent volume of physiological saline and as follows (Yeun-hwa *et al.*, 2005):

Group I: Control (5 mice): treated with 1ml of physiological saline.

Group II: β -glucan treatment (5 mice): treated with 1ml of β -glucan (200 mg/kg).

Group III: β -glucan treatment (5 mice): treated with 1ml of β -glucan (400 mg/kg).

Group IV: β -glucan treatment (5 mice): treated with 1ml of β -glucan (800 mg/kg).

At the tenth day, the mice were sacrificed; tumor samples were collected and preserved in formalin (10%) for histological sectioning.

2.2.10 Histological Analysis of Tumors

Sections were first isolated and cleaned from excess 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 (Bankroft, 1980). 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 °C then were stick in a glass slide in a proper angled manner to obtain the best result, then glass slides containing 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 hematoxylin and eosin stain (Junquera *et al.*, 2005; Humson, 2001).

2.2.11 Statistical analysis

Data were analyzed statistically using SPSS program version 20. Results were expressed using simple statistical parameters such as mean and standard error. Differences between means were assessed by ANOVA, followed by either LSD or Duncan test. Acceptable level of significance was considered to be $P < 0.05$.

Chapter Three

Results and Discussion

3. Result and Discussion

3.1 β -glucan from *P.eryngii*

In this study, β -glucan was extracted using a method described by (Wood, 1993) with simple manipulation, which depended on hot water extraction steps. Proteins in the extract were removed by using HCl. The reason behind using Hcl was to remove proteins and other impurities. These impurities which may found with glucan affect its physical and chemical properties and may cause reducing in its ability for water absorptions and then it became unsuitable for some applications like food industry (James *et al.*, 1991).

The polysaccharide was precipitated using ethanol absolute and the total polysaccharide was estimated by the phenol-sulfuric method (DuBois *et al.*, 1956). However, a study done by Kanagasabapathy and his colleagues (2012) also extracted the polysaccharides from the grey oyster mushroom using hot-water extraction method and found approximately 80% β -glucan in their crude extract.

Additional studies reported that the branched β (1 $\rightarrow$ 3) and β (1 $\rightarrow$ 6)-D-glucan is predominated in the hot water polysaccharide extract from *Pleurotus ostreatus* and *Pleurotus eryngii* (Synytsya *et al.*, 2009).

3.2 Testing of Glucan Structure

3.2.1 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 3.1), which indicating the presence of the extracted glucan. Such peak showed the same retention time of the glucan standard (2.193) (Figure 3.1).

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 (Al-Aubydi and Abed, 2011).

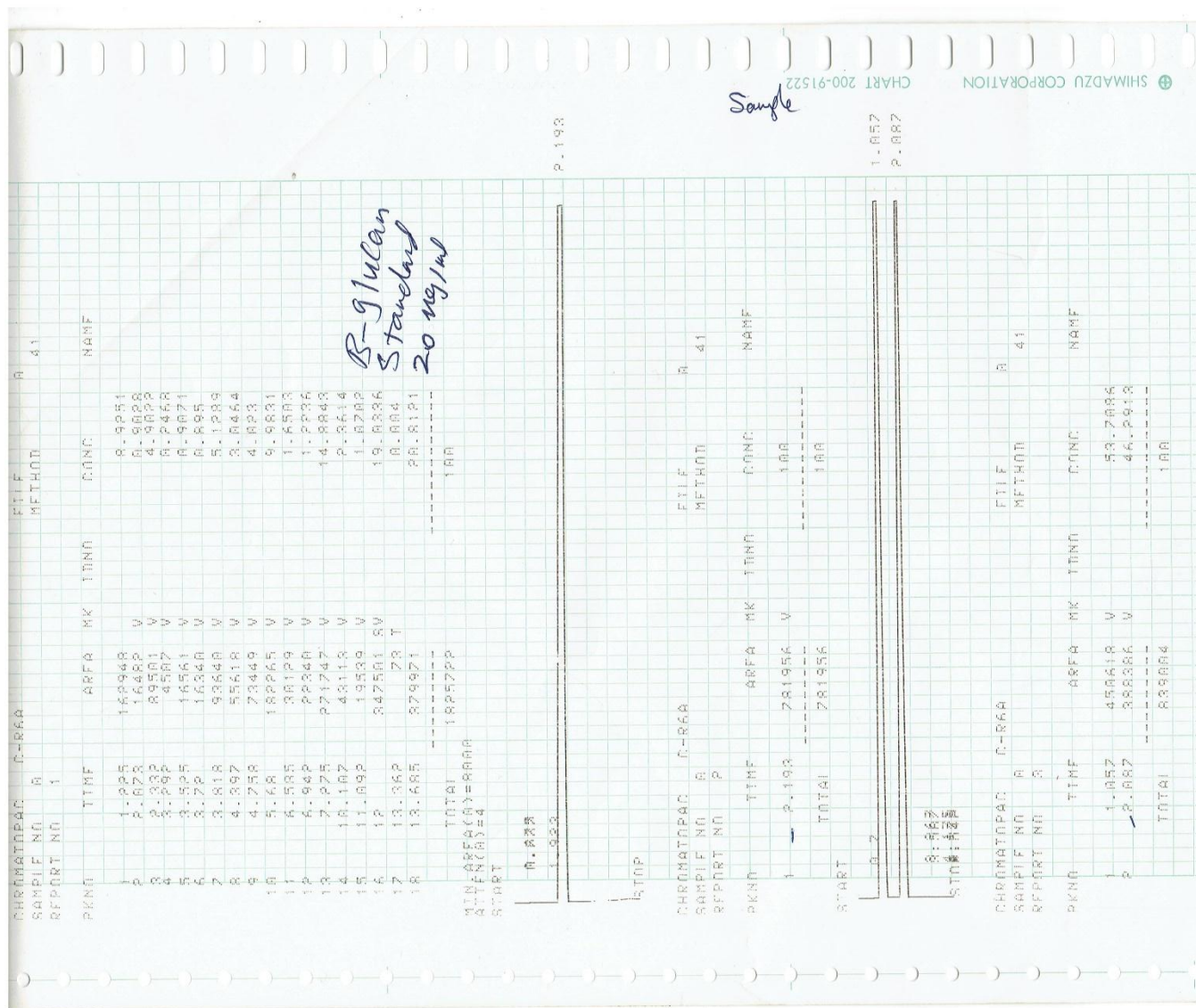


Figure (3.1): HPLC analysis for the *P. eryngii* β -glucan as compared with the standard *Euglena gracilis* β -glucan

3.2.2 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.

Result in (Fig 3.2a) shows 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}) (Fig 3.2b) (Hozova *et al.*, 2007).

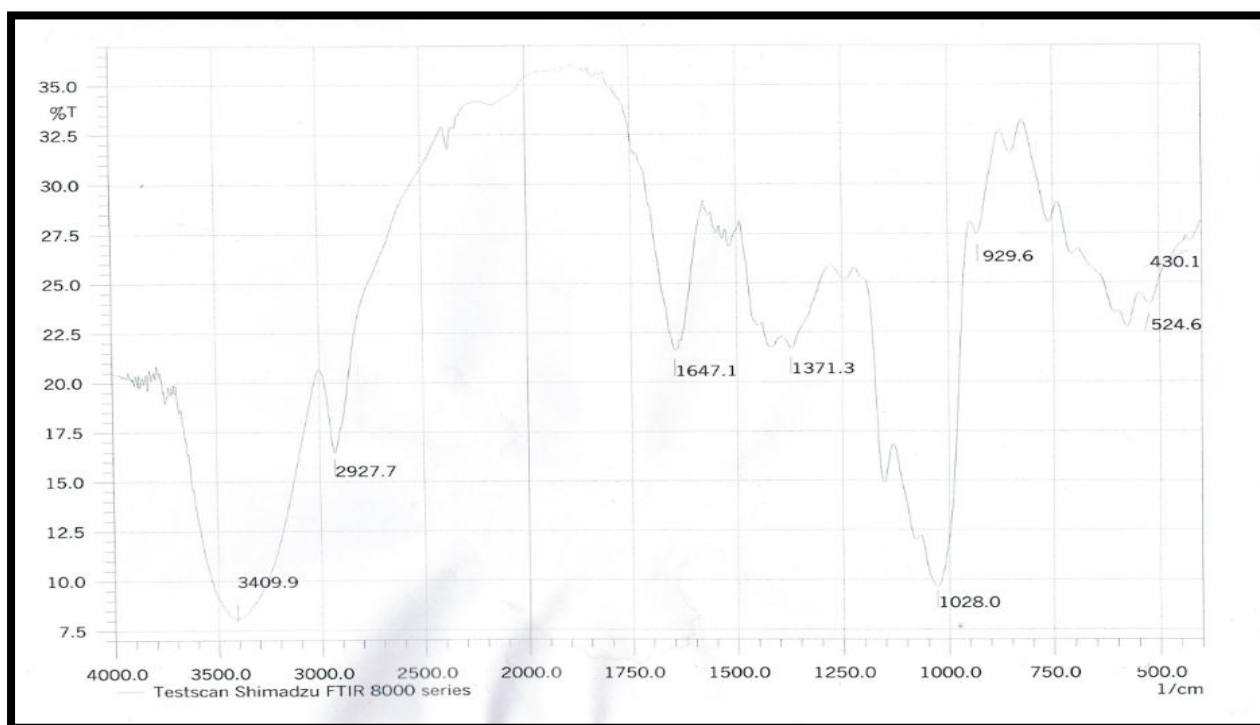


Figure (3.2a): FT-IR analysis for extracted *P. eryngii* β - glucan.

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}) (Karreman *et al.*, 2006). 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 (Ibrahim *et al.*,

2006). 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 .

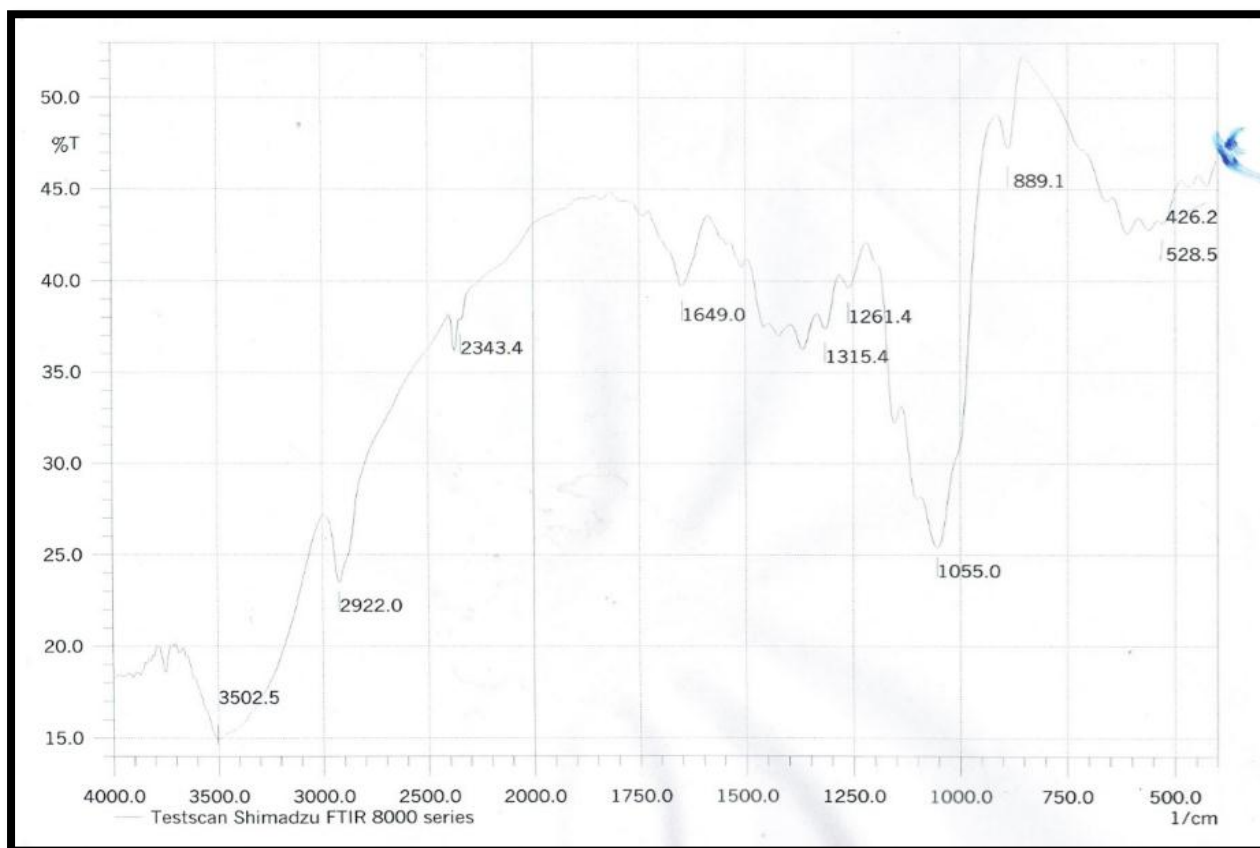


Figure (3.2b): FT-IR analysis for standard *P. eryngii* β -glucan

3.2.3 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.* (1956) depending on the standard curve of glucose, and estimating the protein contents according to Bradford, (1976) depending on the standard curve of bovine serum albumin. Accordingly, the carbohydrate content was (250 mg/ml), while the protein concentration was very low (0.04 mg/ml). 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, (2002) 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 $\rightarrow$ 3), (1 $\rightarrow$ 6)- β -glucans and linear (1 $\rightarrow$ 3)- α -glucans, but some are true heteroglycans.

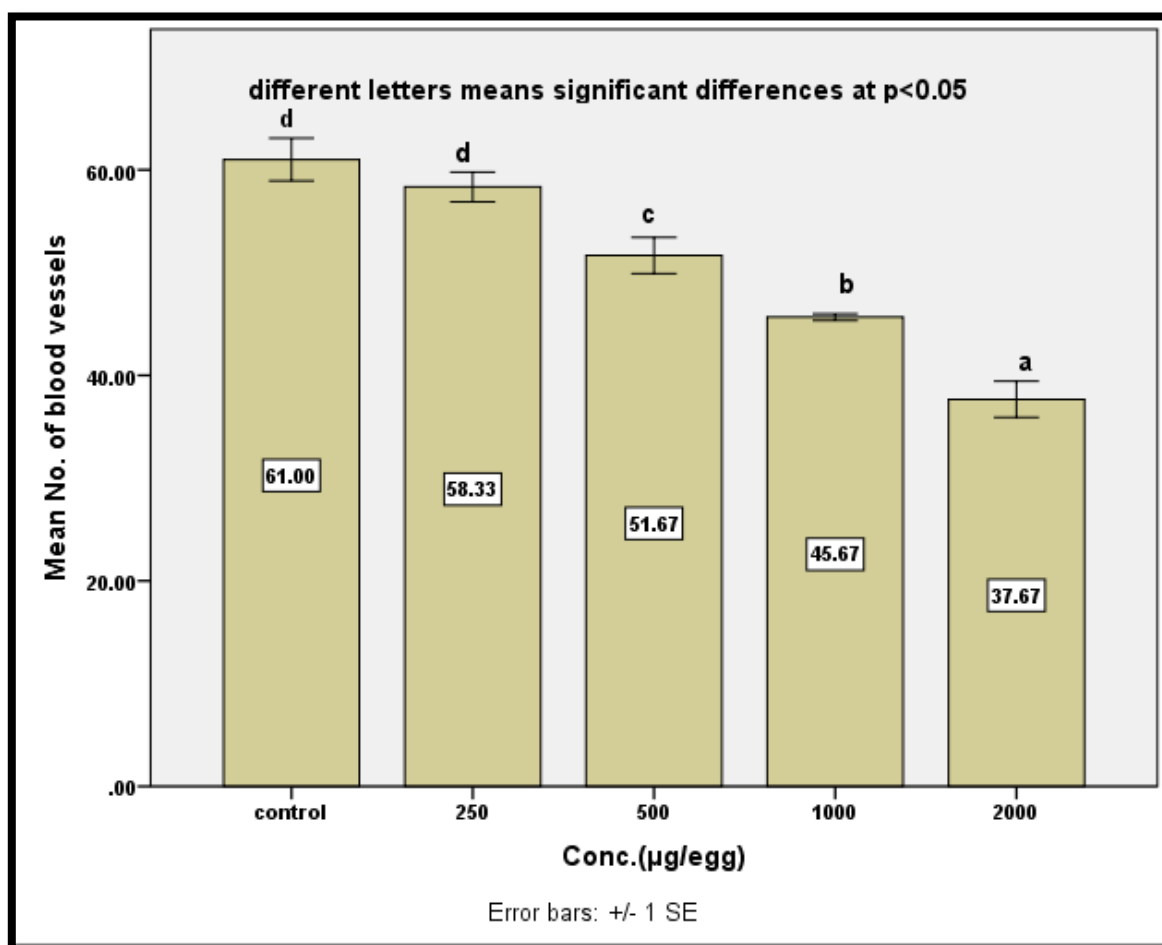
Vetvicka and Vetvickova, (2007) have reported that the glucan purity is an important character in determination of its application since glucan may be used in pharmaceutical, cosmetics, food and other felids.

3.3 Anti-angiogenic Effect of *P. eryngii* β -Glucan

The anti-angiogenic effect of the *P. eryngii* β -glucan was detected by CAM assay. Results in (Fig 3.3) revealed that the dose 250 μ g/egg of β -glucan showed a no significant inhibition effect on neovascularization and the number of blood vessels branches was (58.33 $\pm$ 1.45) as compared with the negative control (61.0 $\pm$ 2.08) as shown in figure (3.4 a and b).

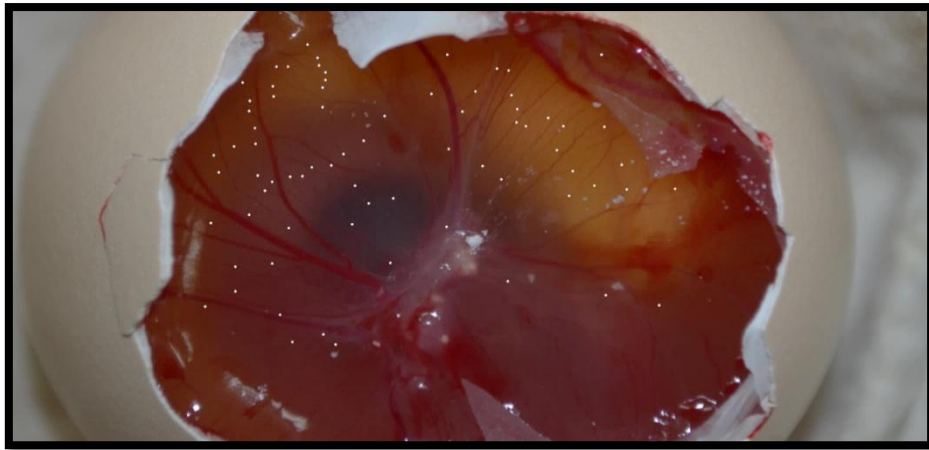
While the doses (500 and 1000 μ g/egg) showed a significant inhibition of the neovascularization as compared with the negative control and revealed a decreased number of blood vessels branches (51.67 $\pm$ 1.76 and 45.67 $\pm$ 0.33) respectively as shown in Figure (3.3 and 3.4 c, d).

Figure (3.3): Effect of β -glucan on the neovascularization of the chick embryo

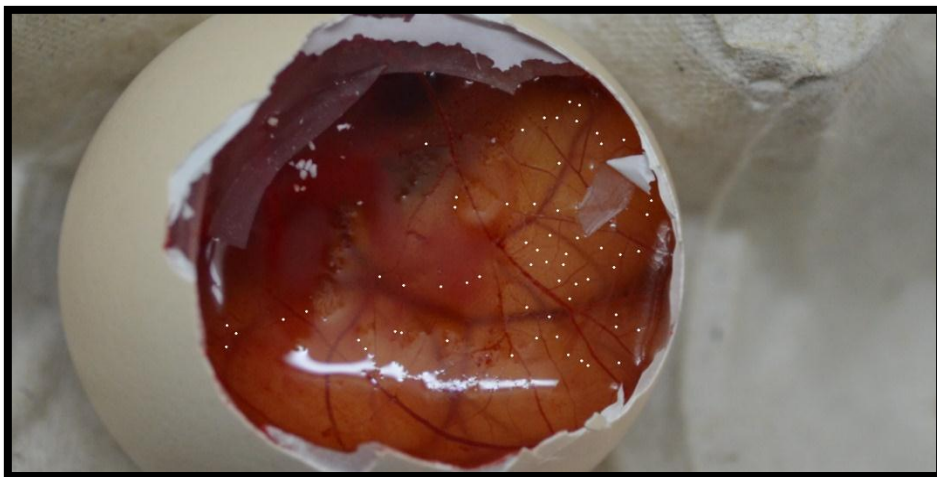


Different Letters ^{a,b,c,d} refers to significant results among groups

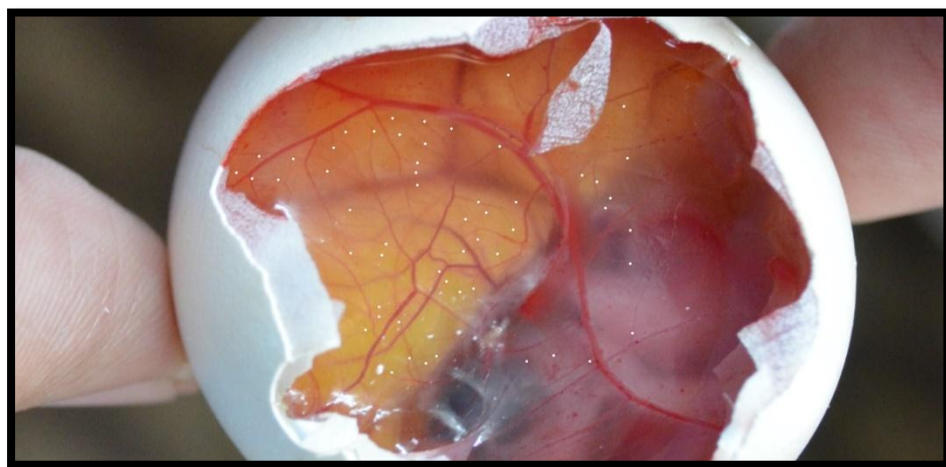
On the other hand, it was noticed that when the dose of β -glucan was increased to 2000µg/egg, CAM capillaries were dramatically decreased upto 50% (37.67 ± 1.76) as shown in (Fig 3.4e). This result revealed that due to an increase in the concentration of β -glucan, This will cause a decrease in the chick CAM angiogenesis.



(A)



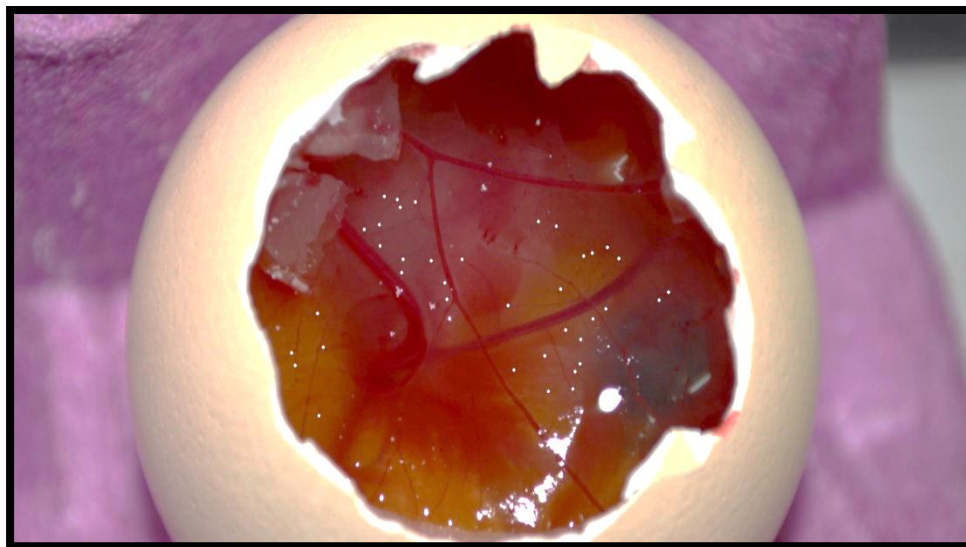
(B)



(C)



(D)



(E)

Figure (3.4): The effect of *P. eryngii* β -glucan concentrations on the neovascularization of the chick embryo blood vessels

A-Control B-250 μ g/egg C-500 μ g/egg

D-1000 μ g/egg E-2000 μ g/egg

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

The overall results indicated that β -glucan extracted from *P. eryngii* affects the generation of the new blood vessels and hence reduces the number of blood capillaries. The decrease of the neovascularization may be attributed to several lines of evidence that support the role of β -glucan as an anti-angiogenic factor.

Angiogenesis is involved in many physiological processes, but also is a hallmark in the pathology of many diseases (cancer, ischemia, atherosclerosis, inflammatory diseases), in wound healing and in tissue regeneration. It is of crucial importance for the survival of cancer cells, for tumor growth and spreading. Angiogenesis is a complex process, tightly regulated at molecular level by involving growth factors, receptors, extracellular matrix (ECM) proteins and the humoral factors (Dimova *et al.*, 2014).

β -glucan is most effective in promoting the secretion of IL-12 that has been shown to directly inhibit angiogenesis of endothelial cells (Del Vecchio *et al.*, 2007), many studies revealed that β -1,3-D-Glucan derived from *S. crispa* (edible mushroom with medicinal properties) is reported to stimulate interferon (IFN)- γ and IL-12p70 production from mice spleen cells (Yamamoto *et al.*, 2009). Several cytokines such as tumor necrosis factor (TNF)- α , IFN- α , IFN- γ , IL-12, and interferon-inducible protein 10 (IP-10) are known anti-angiogenic factors (Harada *et al.*, 2002).

The VEGF is a factor 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 (Xu *et al.*, 2016).

VEGF family members are the most important factors that induce angiogenesis. Number of essential properties is attributed to VEGF with direct effect on promoting angiogenesis. First, it is its proliferative effect on target endothelial cells, which start growing under its influence, increasing their

survival and decreasing the apoptotic rate. Second, it enhances vascular permeability which is connected to extravasation and migration of different cells from/into circulation (Motzer *et al.*, 2009).

3.4 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 intraperitoneally with (200, 400 and 800 mg/kg) of β -glucan and control.

Tumor section of normal mice control showing pleomorphic malignant cells which arranged as glandular structure (adenocarcinoma) (Fig 3.5).

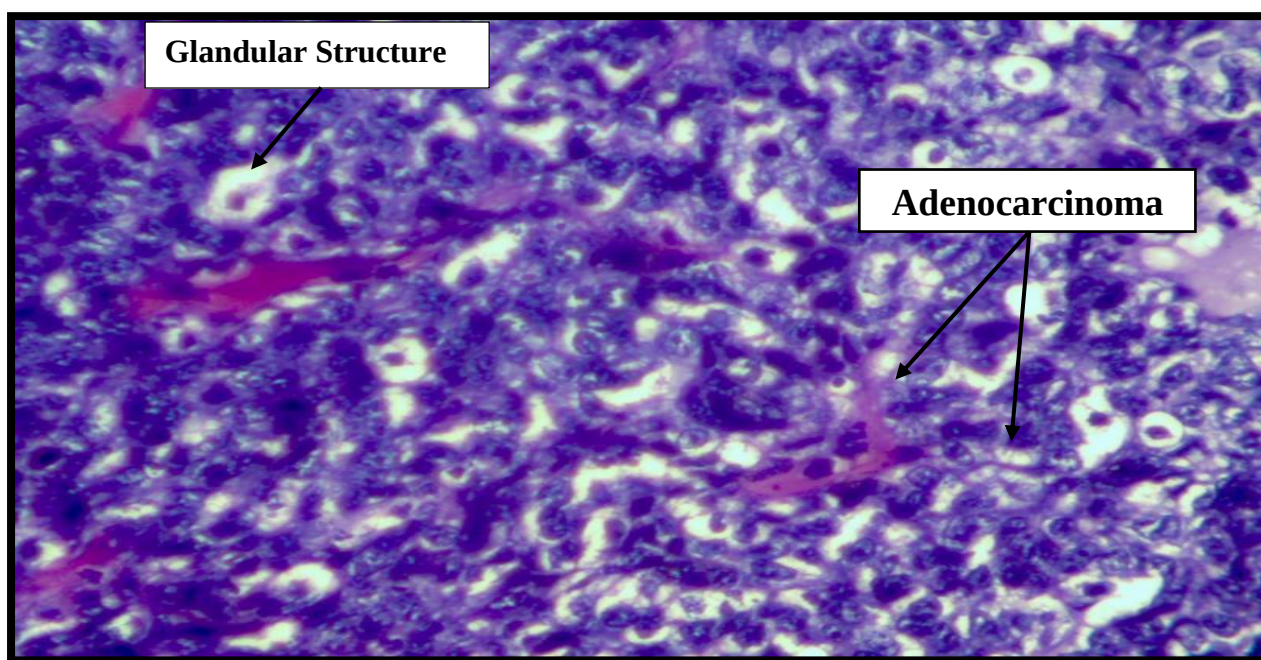


Figure (3.5): Tumor section of mice bearing AN3 murine mammary adenocarcinoma showing pleomorphic malignant cells which are arranged as glandular structure (adenocarcinoma) (arrows) (H and E; 400X).

Tumor section of mice (200 mg/kg) showing a beginning necrosis of the malignant cells (Fig 3.6).

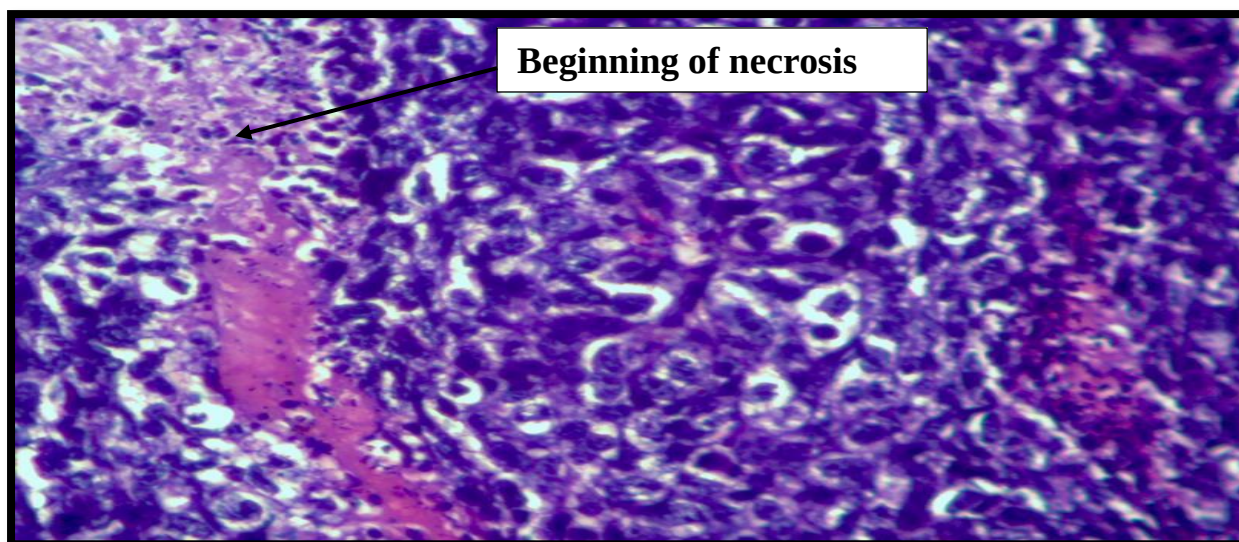


Figure (3.6): Tumor section of AN3 murine adenocarcinoma mice treated with (200 mg/kg) extracted β -glucan showing a beginning necrosis of the malignant cells (arrows) (H and E; 400X).

Tumor section of mice (400 mg/kg) showing wide area of necrosis and inflammatory cells (Fig 3.7).

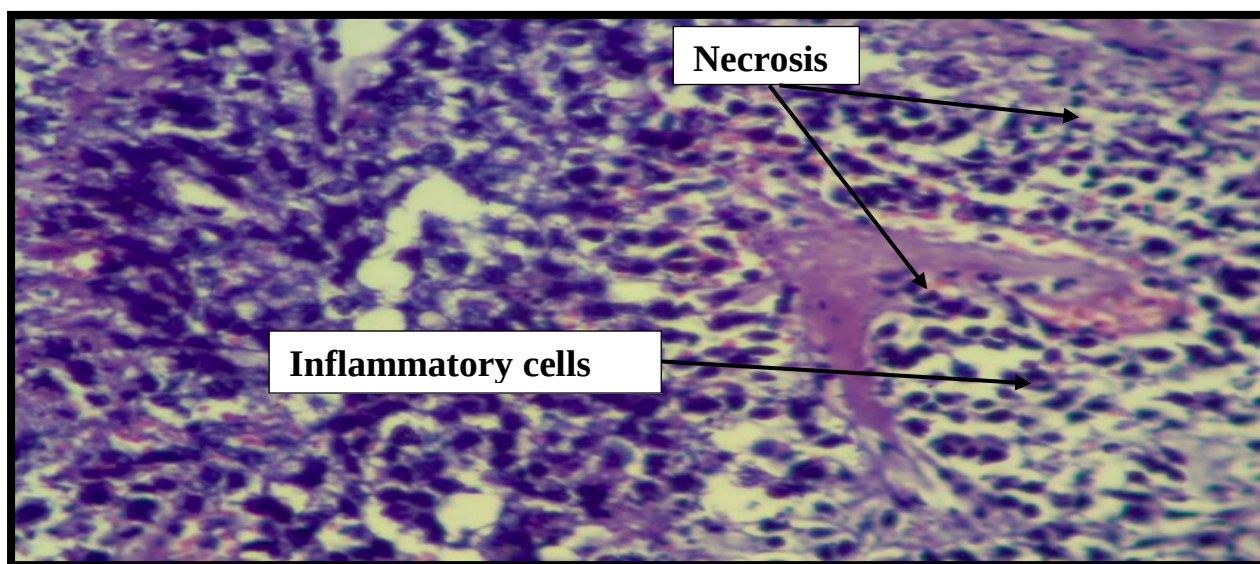


Figure (3.7): Tumor section of AN3 murine adenocarcinoma mice treated with (400 mg/kg) extracted β -glucan mice showing wide area of necrosis and inflammatory cells (arrows) (H and E; 400X).

Tumor section of mice (800 mg/kg) showing inflammatory cells and more prominent appearance of necrosis of malignant cells (Fig 3.8).

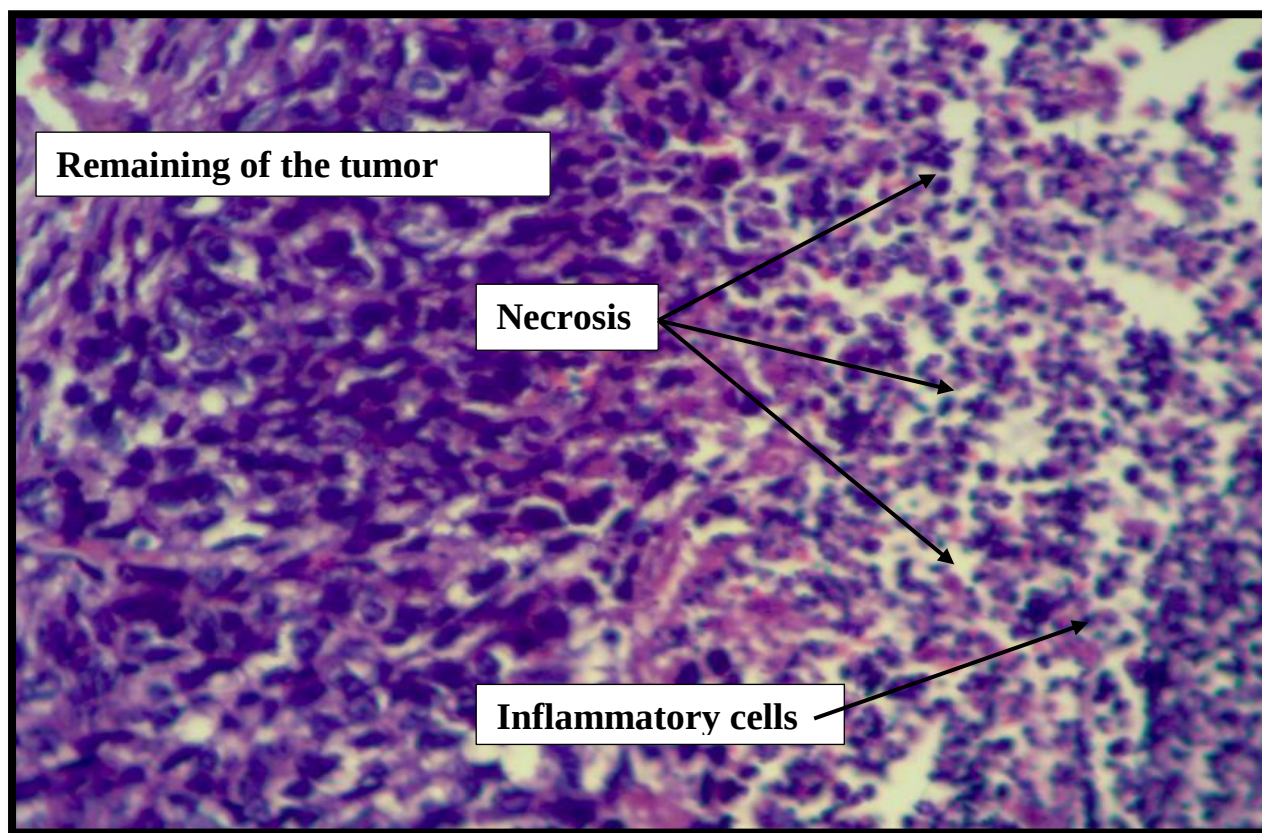


Figure (3.8): Tumor section of AN3 murine adenocarcinoma mice treated with (800 mg/kg) extracted β -glucan showing inflammatory cells and more prominent appearance of necrosis of malignant cells (arrows) (H and E; 400X)

The histopathological results indicated that the β -glucan extracted from medicinal mushroom *P. 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 (Yeun-hwa *et al.*, 2005).

Sparassis crispa (Hanabiratake in Japanese), is an edible and medicinal mushroom containing large amount of β -1,3-D-glucan. *Sparassis crispa* 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 (Yamamoto *et al.*, 2009).

Many 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 (Yang *et al.*, 2013).

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 (Yoon *et al.*, 2008).

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 (Xu *et al.*, 2016).

Chapter Four

Conclusions and Recommendations

Conclusions and Recommendations

4.1 Conclusions:

- Hot water extraction of β -glucan is the optimum method for obtaining β -glucan in high yield.
- β -glucan exhibited an anti-angiogenic effect in a dose dependent manner.
- β -glucan also showed the ability to act as an anti-tumor factor

4.2 Recommendations:

- Studying the ability to involve β -glucan from medicinal mushrooms in food application.
- Studying the promising aspects of using β -glucan as a radioprotection therapy for cancer patients receiving radiotherapy.
- Evaluate the antioxidant effect of β -glucan.
- Studying the cytotoxic effect of β -glucan on other tumor cells *in vivo* and *in vitro*.

References

References

- **Al-Aubydi, M.** and Abed, R. M. (2011). The usage of β -glucan extracted from local mushroom as immunomodulator. *Curr. Res. J.Biol.Sci.* 3(5):535-541.
- **Al-Shammari, A.,** Yaseen, Y. and Alwan, M.J (2008): Establishment and characterization of AN3 first murine mammary adenocarcinoma transplantable tumor line in Iraq. *Iraqi Journal of Cancer.* 1(2):1.
- **Al-Shammari, A.M.,** Yaseen, N.Y. and Alwan, M.J (2011): Immunology Study for NDV Treatment in Mice Bearing Mammary Adenocarcinoma Tumor. *Iraqi Journal of Cancer and Medical Genetics.* 4(1):11.
- **Babicek, K.;** Cechova, I.; Simon, R. R.; Harwood, M.; and Cox, D. J. (2007). Toxicological assessment of a particulate yeast (1, 3/1, 6) d-glucan in rats. *Food and Chemical Toxicology.* 45: 1719–1730.
- **Bankroft, L. (1980):** Basic histological techniques; 4th ed; an Arboer science publishers. Pp: 130-145.
- **Beauvais A,** Bruneau JM, Mol PC, Buitrago MJ, Legrand R. and Latge JP. (2000). The glucan synthase complex of *Aspergillus fumigatus*. *J Bacteriol* 83:2273–2279.
- **Bernard M.** and Latge JP. (2001). *Aspergillus fumigatus* cell wall: composition and biosynthesis. *Med Mycol Suppl* 1 39:9–17.
- **Bohn, J. A., & BeMiller, J. N.** (1995). (1 - 3)- β -D-glucans as biological response. *Carbohydrate Polymers,* 28, 3–14.
- **Borchers, A. T.;** Keen C.L. and Gershwin ME, (2004). Mushrooms, tumors, and immunity: an update. *Experimental Biology and Medicine* .229: 393–406.
- **Bradford, M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annu.Biochem.* 72: 248-254.

- **Brown GD.** (2006). Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol.* 6:33–43. doi: 10.1038/nri1745. [PubMed] [Cross Ref].
- **Buller, C. S., & Voepel, K. C.** (1990). Production and purification of an extracellular polyglucan produced by *Cellulomonas flavigena* strain KU. *Journal of Industrial Microbiology*, 5, 139-145.
- **Carbonero, E. R.,** Gracher, A. H. P., Smiderle, F. R., Rosado, F. R., Sasaki, G. L., Gorin, P. A. J., & Iacomini, M. (2006). A β -glucan from the fruit bodies of edible mushrooms *Pleurotus eryngii* and *pleurotus ostreatoroseus*. *Carbohydrate Polymers*, 66, 252- 257.
- **Carla C,** Daris F, Cecilia B, Francesca B, Francesca C, Paolo F. (2012). Angiogenesis in head and neck cancer: A review of literature. *Journal of oncology*. ID 358472.
- **Chang,** Shu-ting; Miles and Philip G. (2004). "Pleurotus – A Mushroom of Broad Adaptability". *Mushrooms: cultivation, nutritional value, medicinal effect, and environmental impact* (2nd ed.). CRC Press. pp. 315–325.
- **Chen XY.** and Kim JY. (2009). Callose synthesis in higher plants. *Plant Signal Behav.* 4:489-92.
- **Chen, J.** and Seviour, R. (2007). Medicinal importance of fungal beta-(1-3), (1-6) glucans. *Mycol. Res.* 111(6): 635-652.
- **Chen, S. C.;** Lu, M.; Cheng. J. and Wang, D. (2005). Antiangiogenic activities of polysaccharides isolated from medicinal fungi. *Microbiol. Lett.* 249(2): 247-254.
- **Cheung, N. K.** and Modak S. (2002). Oral (1/3),(1/4)-b-D-glucan synergizes with antiganglioside GD2 monoclonal antibody 3F8 in the therapy of neuroblastoma. *Clinical Cancer Research* 8: 1217– 1223.

- **Cheung, N.K.;** Modak, S.; Vickers, A. and Knuckles, B., (2002). Orally administered β -glucans enhance anti-tumor effects of monoclonal antibodies. *Cancer Immunology, Immunotherapy* 51: 557–564.
- **Chu,** and YiFang (2014). *Oats Nutrition and Technology*. Barrington, Illinois: Wiley Blackwell.
- **Clarke, A. E. & Stone, B. A.** (1963). Chemistry and biochemistry of fl-1~3-glucans. *Rev. pure app. Chem. (Aust.)*, 13, 134.
- **Del Vecchio, M.,** Bajetta, E., Canova, S., Lotze, M.T., Wesa, A., Parmiani, G. and Anichini, A., (2007). Interleukin-12: biological properties and clinical application. *Clinical Cancer Research* 13, 4677–4685.
- **Delley PA.** and Hall MN. (1999). Cell wall stress depolarizes cell growth via hyperactivation of RHO1. *J Cell Biol* 147:163–174.
- **Dimova, I,** Popivanov, G, and Djonov, V. (2014). Angiogenesis in cancer – general pathways and their therapeutic implications. *JBUON*; 19(1): 15-2.
- **Di Renzo L,** Yefenof E. and Klein E. (1991). The function of human NK cells is enhanced by beta-glucan, a ligand of CR3 (CD11b/CD18) *European journal of immunology*. 21:1755–1758. doi: 10.1002/eji.1830210726.
- **Douglas CM.** (2001). Fungal β (1,3)-D-glucan synthesis. *Med. Mycol.* 39:55–66.
- **Dubois, M.;** Gilles, K.A.; Hamilton, J.K.; Rebers, P.A. and Smith,F. (1956). Colorimetric methods for determination of sugars and related substance. *Anal.Chem.* 28: 350-356.*Experimental Microbiology*. 1st edition. Mosby-Year Book, Inc.USA.

- **Duda DG**, Sunamura M, Lozonschi L, Kodoma T, Egawa S. and Matsumoto G. (2000). Direct in vitro evidence and in vivo analysis of the antiangiogenesis effects of interleukin -12. *Cancer Res.* 60:1111–16.
- **El-Enshasy HA.** and Hatti-Kaul R. (2013). Mushroom Immunomodulators: unique molecules with unlimited applications. *Trends Biotechnol.* 31(12): 668-77.
- **Ferrara N.** and Davis Smyth T. (1997). The biology of vascular endothelial growth factor. *Endocr Rev.*18:4–25.
- **Fleet, GH.** (1991). Cell walls. In Rose AH, Harrison JS. ed; *The Yeasts*. 2nd edn Vol. 4. London: Academic Press. p 199–277.
- **Folkman J.** (1995). *The Molecular Basis of Cancer*. Philadelphia: WB Saunders. Tumour Angiogenesis. In: Mendelsohn J, Howley PM, Israel MA, Liotta LA, eds; pp. 206–32.
- **Fontaine T**, Simenel C, Dubreucq G, Adam O, Delepierre M *et al.* (2000). Molecular organization of the alkali-insoluble fraction of the *Aspergillus fumigatus* cell wall. *J Biol Chem* 275:27594–27607.
- **Futatsuyama, H.**, Yui, T., & Ogawa, K. (1999). Viscometry of curdlan, a linear (1-3)- β -D-glucan, in DMSO or alkaline solutions. *Bioscience, Biotechnology and Biochemistry*, 63, 1481-1483.
- **Gantner BN**, Simmons RM, Canavera SJ. and Akira S. (2003). Underhill DM. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *The Journal of experimental medicine*. 197:1107–1117. doi: 10.1084/jem.20021787.
- **Gargano ML**, Venturella G and Zervakis GI. (2013). *Pleurotus nebrodensis*: A very special mushroom. Sharjah, U.A.E.: Bentham Science Publishers.
- **Gavin, T.P**; Robinson, C.B; Yeager, R.C; England, J.A; Nifong and L.Wand Hickner, R.C (2004). "Angiogenic growth factor response to

- acute systemic exercise in human skeletal muscle. *Journal of Applied Physiology* 96 (1): 19–24.
- **Gerber HP**, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V. and Ferrara N. (1998) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3 -kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J biol. Chem.* 273(46):30366–43.
 - **Giatromanolaki A.**, Koukourakis M., O'Byrne K., Fox S., Whitehouse R., Talbot D.C., Harris A.L. and Gatter K.C. (1996) Prognostic value of angiogenesis in operable non-small cell lung cancer. *J. Pathol.* 179, 80–88. CrossRef Medline Web of Science.
 - **Goodridge HS**, Simmons RM and Underhill DM. (2007). Dectin-1 stimulation by *Candida albicans* yeast or zymosan triggers NFAT activation in macrophages and dendritic cells. *J Immunol.* 178:3107–3115.
 - **Gore, R., & Miller, K.** (1993). Cyclic β -1,6-1,3 glucans are synthesized by *Bradyrhizobium japonicum* bacterioids within soybean (*Glycine max*) root nodules. *Plant Physiology*, 102, 191-194.
 - **Griscelli F.** and Li H. (1998) Bennaceur-Griscelli A, Soria J, Opolon P, Soria C, et al. Angiostatin gene transfer; inhibition of tumour growth in vivo by blockage of endothelial cell proliferation associated with a mitosis arrest. *Proc Natl Acad Sci USA.* 95:6367–72.
 - **Grun CH**, Hochstenbach F, Humbel BM, Verkleij AJ, Sietsma JH *et al.* (2005). The structure of cell wall α -glucan from fission yeast. *Glycobiology* 15:245–257.
 - **Gummadi, S. N., & Kumar, K.** (2005). Production of extracellular water insoluble β -3-glucan (curdlan) from *Bacillus* sp SNC07. *Biotechnology and Bioprocess Engineering*, 10, 546-551.

- **Gupta MK.** and Qin RY. (2003). Mechanism and its regulation of tumour induced angiogenesis. *World journal of gastroenterol.* 9(6):1144–55.
- **Hajitou A,** Grignet C, Devy L, Berndt S, Blacher S, Deroanne CF, *et al.* (2002). The antitumoural effect of endostatin and angiostatin is associated with a downregulation of vascular endothelial growth factor expression in tumour cells. *FASEB J.* 16:1802–04.
- **Han,** and Man Deuk (March 2008). "Solubilization of water-insoluble β -glucan isolated from *Ganoderma lucidum*". *Journal of Environmental Biology.*
- **Harada T.,** Miura N. N, Adachi Y., Nakajima M., Yadomae T. and Ohno N. (2002). *J. Interferon Cytokine Res.,* 22, 1227—1239.
- **Harada, T.,** Misaki, A., & Saito, H. (1968). Curdlan: a bacterial gel-forming β -D-1,3-glucan. *Archives of Biotechnology and Biophysics,* 124, 292-298.
- **Hasina R.** and Lingen MW. (2011). Angiogenesis and oral cancer. *Journal of Dental Education.* 65(11):1282–90.
- **Hassan S.S.,** Al-Saffar A.Z., Ibrahim H.K. (2014). Antiangiogenic and immunological effect of β -glucan extracted from *Saccharomyces cerevisiae* HS-1. *International Journal of Biotechnology.* Photon 112, 365-373.
- **Herre J,** Gordon S, and Brown GD. (2004). Dectin-1 and its role in the recognition of beta-glucans by macrophages. *Molecular immunology.* 40:869–876. doi: 10.1016/j.molimm.2003.10.007.
- **Herre J,** Marshall AS, Caron E, Edwards AD, Williams DL, Schweighoffer E, Tybulewicz V, Reis e Sousa C, Gordon S, and Brown GD. (2004). Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood.* 104:4038–4045. doi: 10.1182/blood-2004-03-1140.

- **Ho, J. C.,** Konerding, M. A. *et al.* (2004). Fungal polysaccharopeptide inhibits tumor angiogenesis and tumor growth in mice. *Life Sciences* 75(11), 1343–1356.
- **Hong F,** Yan J, Baran JT, Allendorf DJ, Hansen RD, Ostroff GR, Xing PX, Cheung NK, and Ross GD. (2004). Mechanism by which orally administered beta-1,3-glucans enhance the tumoricidal activity of antitumor monoclonal antibodies in murine tumor models. *J Immunol.* 173:797–806.
- **Hori A,** *et al.* (1991). Suppression of solid tumour growth by immune neutralising monoclonal antibody against human fibroblast growth factor. *Cancer Res.* 51:6180–84.
- **Hozova, B.,** Kuniak, L., Moravckov, P., and Gajdošová, A. (2007). Determination of water-insoluble β -D-glucan in the whole- grain cereals and pseudocereals. *Czech J. Food Sci.,* 25:316-324.
- **Huang S,** Ullrich SE, and Bar-Eli M. (1991). Regulation of tumour growth and metastasis by interleukin-10: the melanoma experience. *J Interferon Cytokine Res.* 19:697–703.
- **Ibrahim, M.;** Alaam, M.; EI-Haes, H., Jalbout, J. F.; and Leon, A. (2006). Analysis of the structure and vibrational spectra of glucose and fructose. *Ecl. Quim., Sao Paulo,* 31(3): 15-21.
- **Inon de Iannino, N.,** & Uglalde, R. A. (1993). Biosynthesis of cyclic β -(1-3), β -(1-6) glucans in *Bradyrhizobium* spp. *Archives of Microbiology,* 159, 30-38.
- **Jamas, S.,** Rha, C. and Sinskey, A. J. (1991). Glucan composition and process for preparation thereof. United States Patent, No.5,033,972.
- **Jing X,** Mao D, Geng L, Xu C (2013) Medium optimization, molecular characterization and bioactivity of exopolysaccharides from *Pleurotus eryngii*. *Arch Microbiol* 195: 749-757.

- **Jonasch E**, and Haluska FG. (2001). Interferon in oncological practice: review of interferon biology, clinical applications and toxicities. *Oncologist*. 6:34–55.
- **Junquera, L.** and Carneiro, J.R.O. (2005). Male Reproductive System. In: *Basic Histology*. Janson, M.; Harriet and Petery J., Stamford. 11th ed. Pp: 418-434.
- **Kanagasabapathy, G.**, Kuppusamy, U.R., Abd Malek, S.N., Abdulla, M.A., Chua, K.H. and Sabaratnam, V. (2012). Glucan-rich polysaccharides from *Pleurotus sajorcaju* (Fr.) Singer prevents glucose intolerance, insulin resistance and inflammation in C57BL/6J mice fed a high-fat diet. *BMC Complementary and Alternative Medicine*, 12: 261.
- **Kapteyn JC**, Van Den Ende H. and Klis FM. (1999). The contribution of cell wall proteins to the organization of the yeast cell wall. *Biochim Biophys Acta* 1426:373–383.
- **Karamysheva AF**. (2008). Mechanism of angiogenesis. *Biochemistry*. 73:935–48.
- **Karreman, R. J.**, Dague, E., Gaboriaud, F., Guiles, F., Duval J. F., and Lindsey, G. G. (2006). The stress response protein Hsp12p increase the flexibility of the yeast *Sacchromyces cerevisiae*. *Biochim. Biophys. Acta*. 1436:239-246.
- **Kenyon, W. J.**, & Buller, C. S. (2002). Structural analysis of the curdlan-like exopolysaccharide produced by *Cellulomonas flavigena* KU. *Journal of Industrial Microbiology and Biotechnology*, 29, 200-203.
- **Kenyon, W. J.**, Esch, S. W., & Buller, C. S., (2005). The curdlan-type exopolysaccharide produced by *Cellulomonas flavigena* KU forms part of an extracellular glycocalyx involved in cellulose degradation. *Antonie van leewenhoek*, 87, 143-148.

- **Kim DJ, et al.**(1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour cell growth in vivo. *Nature*. 362:841–44.
- **Kim YM, Hwang S, Kim YM, Pyun BJ, Kim TY, Lee ST, et al.** (2002). Endostatin blocks vascular endothelial growth factor mediated signalling via direct interaction with KDR/Flk-1. *J Biol Chem*. 2002;277:27872–79.
- **Kiss JZ, Vasconcelos AC. And Triemer RE.** (1987). Structure of the euglenoid storage carbohydrate, paramylon. *American J Botany* 74:877-82.
- **Klein E, Di Renzo L. and Yefenof E.** (1990). Contribution of CR3, CD11b/CD18 to cytolysis by human NK cells. *Molecular immunology*. 27:1343–1347. doi: 10.1016/0161-5890(90)90041-W.
- **Kohn S, Nagy JA, Dvorak HF. and Dvorak AM.** (1992). Pathways of macromolecular tracer transport across venules and small veins. Structural basis for hyperpermeability of tumour blood vessels. *Lab Invest*. 67:596–607.
- **Kollar, R, Reinhold BB, Petrakova E, Yeh HJ, Ashwell G. and Drgonova J.** (1997). Architecture of the yeast cell wall. b(1,6)-glucan interconnects mannoprotein, b(1,3)-glucan, and chitin. *J Biol Chem* 272:17762–17775.
- **Kong W-S.** (2004). Mushroom growers handbook 1: Oyster mushroom cultivation 1: 54-61.
- **Koukourakis M.I., Giatromanolaki A., Fountzilas G., Sivridis E., Gatter K.C. and Harris A.L.** (2000) Angiogenesis, thymidine phosphorylase, and resistance of squamous cell head and neck cancer to cytotoxic and radiation therapy. *Clin. Cancer Res*. 6, 381–389.
- **Kues, U., and Liu, Y.** (2000). Fruiting body production in basidiomycetes, *Applied microbiology and biotechnology*, 54, 141-152.

- **Kumar V**, Abbas AK. and Fausto N. (2004). Robbins and Cotran Pathologic basis of disease. 7th ed. Philadelphia: Saunders; Elsevier Inc.
- **Laroch, C.** and Michaud, P. (2007). New Developments and Prospective Applications for Beta (1, 3) Glucans. Recent Patents on Biotechnology. 1(1):59-37.
- **Lee, J. N.** ; Lee, D. Y., Ji, I. H. , Kim, G. E , Kim, H. N. ; Sohn, J., Kim, S. and Kim, C.W. (2001). Purification of soluble β -Glucan with immune – enhancing activity from the cell wall of yeast . Biosci. Biotechnol. Biochem. 65(4):837-841.
- **Leung DW**, Cachianes G, Kuang WJ, Goeddel DV. and Ferrara N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 246:1306–09.
- **Li, M. H.;** Miao, Z. H.; Tan, W. F.; Yue, J. M.; Zhang, X. W. and Ding, J. (2004). Pseudolaric acid B inhibits angiogenesis and reduces hypoxia-inducible factor 1 by promoting proteasome-mediated degradation. Clinical Cancer Research 10, 8266-8274.
- **Lin YL**, Liang YC, Lee SS. and Chiang BL. (2005). Polysaccharide purified from *Ganoderma lucidum* induced activation and maturation of human monocyte-derived dendritic cells by the NF-kappaB and p38 mitogen-activated protein kinase pathways. J Leukoc Biol. 78:533–543. doi: 10.1189/jlb.0804481.
- **Liu F.;** Ooi V.E. and Fung M.C. (1999). Analysis of immunomodulating cytokine mRNAs in the mouse induced by mushroom polysaccharides, Life Sci. 64, 1005–1011.
- **Liu Y.** and White PJ. (2011). Molecular weight and structure of water soluble (1→3),(1→4)- β -glucans affect pasting properties of oat flours. J Food Sci 76:C68-74.

- **Mandal S**, Maity KK. and Bhunia SK. (2010). Chemical analysis of new water-soluble (1→6),(1→4)- α,β -glucan and water insoluble (1→3),(1→4)- β -glucan (Calocyban) from alkaline extract of an edible mushroom, *Calocybe indica* (Dudh Chattu). *Carbohydr Res.* 345:2657-63.
- **Manners DJ**, Masson AJ. and Patterson JC. (1973). The Structure of β -1,3-D-glucan from yeast cell walls. *Biochem J* 135:19–30.
- **Mantovani, M.**; Bellini, M. F.; Angeli, J. F.; Oliveira, R. J.; Silva, A. F. and Ribeiro, L. R. (2008). β -Glucans in promoting health: Presentation against mutation and cancer. *Mutation Rese.* 658(3): 154-161.
- **Manzi, P.**, Gambelli, L., Marconi, S., Vivanti, V., & Pizzoferrato, L. (1999). Nutrients in edible mushrooms: an inter-species comparative study. *Food Chemistry*, 65, 477–482.
- **Manzi, P.**, Marconi, S., Aguzzi, A., and Pizzoferrato, L. (2004). Commercial mushrooms: nutritional quality and effect of cooking. *Food Chem.*, 84: 201–206.
- **Marchessault RH.** and Deslandes Y. (1979). Fine structure of (1→3)- β -D-glucans: curdlan and paramylon. *Carbohydr Res* 1979;75:231-42.
- **Mattila, P.**, Suonpää, K., & Piironen, V. (2000). Functional properties of edible mushrooms. *Nutrition*, 16, 694–696.
- **Mayell, M.** and Maitake, A. (2001). Extracts and their therapeutic potential. *Altern. Med. Rev.* 6(1): 48-60.
- **McCann, M. C.** ; Hammouri, M. ; Wilson, R. ; Belton, P., and Roberts, K. (1992). 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* 19: 175–183.

- **Mcintosh, M** (2004). "Curdlan and other bacterial (1→3)-β-D-glucans". Applied Microbiology and Biotechnology.
- **Miller, K. j.**, Gore, R. S., Johnson, R., Benesi, A. J., & Reinhold, V. N. (1990). Cell association oligosaccharides of *Bradyrizobium* spp. Journal of Bacteriology, 172, 136-142.
- **Mizuno, T.** (1999). The extraction and development of antitumour-active polysaccharides from medicinal mushrooms in Japan. International Journal of Medicinal Mushrooms 1: 9-29.
- **Moser TL**, Stack MS, Asplin I, Enghild JJ, Hojrup P, Everitt L, *et al.* (1999). Angiogenesis binds ATP synthase on the surface of human endothelial cells. Proc Natl Acad Sci USA. 96:2811–16.
- **Motzer J**, Hutson TE , Tomczak P et al. (2009). Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med; 356:115-124.
- **Nakanishi, L.**, Kimara, K., Suzuki, T., Ishikawa, M., Banno, L., Sakane, T., & Harada, T. (1976). Demonstration of curdlan-type polysaccharide and some other β-1,3-glucan in microorganisms with aniline blue. Journal of General and Applied Microbiology.
- **Nam, SW.**; Park, JY.; Ramasamy, A.; Shevade,S.; Islam, A.; Long, PM.; Park ,CK.; Park, SE.; Kim, SY.; Lee, SH.; Park, WS.;Yoo, NJ.; Liu, ET.; Miller, LD. and Lee, JY.(2005). Molecular changes from dysplastic nodule to hepatocellular carcinoma through gene expression profiling. Hepatology 42: 809– 818.
- **Nilsson S**, Widmark A. and Norle´n BJ. (2004). A systematic overview of radiation therapy effects in prostate cancer. Acta Oncol. 43: 316/81.
- **Nordin, J. H.** & Kirkwood, S. (1965). Biochemical aspects of plant polysaccharides. A. Rev. PI. Physiol. 16, 393.

- **Norrby, K.** (2006). In vivo models of angiogenesis. *J. Cell. Mol. Med.*, 10, 588–612.
- **Oshikawa K,** Rakhmilevich AL, Shi F, Sondel PM, Yang N and Mahvi DM. (2010). Interleukin 12 gene transfer in to skin distant from the tumour site elicits antimetastatic effects equivalent to local gene transfer. *Hum Gene Ther.* 12:149–60.
- **Ozturk M,** Tel-Cayan G, Muhammad A, Terzioglu P. and Emin Duru M. (2015). Mushrooms: A source of exciting bioactive. *Studies in Natural Products Chem.* 45(1): 363-456.
- **Pelley, R.** and Strickland, F. (2000). Plants, polysaccharides, and the treatment and prevention of neoplasia. *Crit. Rev. Oncog.* 11(34):189-225.
- **Pereyra MT,** Prieto A, Bernabé M, *et al.* (2003). Studies of new polysaccharide from *Lasallia pustulata* (L.) Hoffm. *Lichenologist.* 35:177-85.
- **Purama, R. K.;** Goswami, P.; Khan, A. T. and Goyal, A. (2009). Structural analysis and properties of dextran produced by *Leuconostoc mesenteroides* NRRL B-640. *Carbohydrate Polymers*, 76, 30–35. resistant *Staphylococcus aureus* in rats treated with PGG-glucan is associated with increased leukocyte counts and increased neutrophil oxidative burst activity. *International Journal of Immunopharmacology* 20: 595–614.
- **Ribatti, D.** (2010). The chick embryo chorioallantoic membrane as an in vivo assay to study antiangiogenesis. *Pharmaceuticals.* 3: 482-513.
- **Rice PJ,** Kelley JL, Kogan G, Ensley HE, Kalbfleisch JH, Browder IW. and Williams DL. (2002). Human monocyte scavenger receptors are pattern recognition receptors for (1→3)-beta-D-glucans. *J Leukoc Biol.* 72:140–146.

- **Rogers NC**, Slack EC, Edwards AD, Nolte MA, Schulz O, Schweighoffer E, Williams DL, Gordon S, Tybulewicz VL. and Brown GD. (2005) Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity*. 22:507–517. doi: 10.1016/j.immuni.2005.03.004.
- **Rolin, D. B.**, Pfeffer, P. E., Osman, S. F., Szwergold, B. S., Kappler, F., & Benesi, A. (1992). Structural studies of a phosphocholine substituted β -(1,3;1,6) macrocyclic glucan from *Bradyrhizobium japonicum* USDA 110. *Biochimica et Biophysica Acta*, 1116, 215-225.
- **Rosado, F. R.**, Carbonero, E. R., Kemmelmeier, C., Tischer, C. A., & Iacomini, M. (2002). A partially 3-O-methylated (1 $\rightarrow$ 4)-linked α -D-galactan and α -D-mannan from *Pleurotus ostreatus* Sing. *FEMS Microbiology Letters*, 212, 261–265 *Medicinal Mushrooms*, 1, 69–80.
- **Sato T**, Iwabuchi K, Nagaoka I, Adachi Y, Ohno N, Tamura H, Seyama K, Fukuchi Y, Nakayama H. and Yoshizaki F. (2006). Induction of human neutrophil chemotaxis by *Candida albicans*-derived beta-1,6-long glycoside side-chain-branched beta-glucan. *J Leukoc Biol*. 80:204–211.
- **Schorey JS**, and Lawrence C. (2008). The pattern recognition receptor Dectin-1: from fungi to mycobacteria. *Curr Drug Targets*. 9:123–129. doi: 10.2174/138945008783502430.
- **Sener, G.**; Toklu, H. Z., and Cetinel, S. (2007). β -Glucan protects against chronic nicotine induced oxidative damage in rat kidney and bladder. *Environmental Toxicology and Pharmacology*, 23, 25–32.
- **Senger DR**, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, and Dvorak HF. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*. 219:983–85.
- **Shono T.**, Ono M., Izumi H., Jimi S.I., Matsushima K., Okamoto T., Kohno K., and Kuwano M. (1996) Involvement of the transcription factor

- NF-kappa B in tubular morphogenesis of human microvascular endothelial cells by oxidative stress. *Mol. Cell Biol.* 16, 4231–4239.
- **Sikora, Per** (2012). "Identification of high β -glucan oat lines and localization and chemical characterization of their seed kernel β -glucans". *Food Chemistry*. 33:123-45.
 - **Singh RS**, Saini GK, and Kennedy JF. (2008). Pullulan: microbial sources, production and applications. *Carbohydr Polym.* 73:515-31.
 - **Slaton JW**, Perrotte P, Inoue K, Dinney CP, and Fidler IJ. (1999). Interferon α mediated downregulation of angiogenesis related genes and therapy of bladder cancer are dependent on optimization of biological dose and schedule. *Clin Cancer Res.* 5:2726–34.
 - **Smith JE**, Rowan NJ, and Sullivan R. (2002). *Medicinal Mushrooms: Their therapeutic properties and current medical usage with special emphasis on cancer treatments USA*: University of Strathclyde.
 - **Sun L**, and Zhao Y. (2007). The biological role of dectin-1 in immune response. *International reviews of immunology*. 26:349–364. doi: 10.1080/08830180701690793.
 - **Sutherland, I.W.** (2001). Microbial polysaccharides from gram negative bacteria. *Int. Dairy Res.* 11:663-674.
 - **Synytsya, A.**, Míčková, K., Synytsya, A., Jablonský, I., Spěváček, J., Erban, V., Kovářiková, E. and Čopíková, J. (2009). Glucans from fruit bodies of cultivated mushrooms *Pleurotus ostreatus* and *Pleurotus eryngii*: Structure and potential prebiotic activity. *Carbohydrate Polymers* 76(4): 548-556.
 - **Tada R**, Adachi Y, and Ishibashi K. (2009). An unambiguous structural elucidation of a 1,3- β -D-glucan obtained from liquid-cultured *Grifola frondosa* by solution NMR experiments. *Carbohydr Res.* 344:400-4.

- **Takaku, T.** Kimura, Y. and Okuda, H. (2001). Isolation of an antitumor compound from *Agaricus blazei* Murill and its mechanism of action. *Journal of Nutrition* .131: 1409–1413.
- **Taylor PR,** Brown GD, Reid DM, Willment JA, Martinez-Pomares L, Gordon S, and Wong SY. (2002). The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *J Immunol*. 169:3876–3882.
- **Vetvicka V.;** Thornton B.P. and Ross G.D. (1996) Soluble beta-glucan polysaccharide binding to the lectin site of neutrophil or natural killer cell complement receptor type 3 (CD11b/CD18) generates a primed state of the receptor capable of mediating cytotoxicity of iC3b-opsonized target cells, *J. Clin. Invest*. 98, 50–61.
- **Vetvicka, V.** and Yvin, J. (2004). Effects of marine β -1,3 glucan on immune reactions. *International Immunopharmacology*. 4: 721– 730.
- **Vetvicka, V.** and Vetvickova, J. (2007). An evaluation of the immunological activities of commercially available β -(1,3) glucans. *JANA*, 10:25-31.
- **Volman,** Julia J (2007). "Dietary modulation of immune function by β -glucans". *Physiology & Behaviour*.
- **Wang S.Y.;** Hsu M.L.; Hsu H.C., Tzeng C.H., Lee S.S.; Shiao M.S. and Ho C.K. (1997) The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes, *Int. J. Cancer*, 70, 699–705.
- **Wasser S.P.** (2002): Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, 60: 258–274.

- **Wasser, S.P.**, and Weis, A.L. (1999). Medicinal properties of substances occurring in higher Basidiomycetes mushrooms: current perspectives (review). *Int. J. Med. Mushrooms*, 1: 31–62.
- **Wood P.J.** (1993). 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. pp. 83-112.
- **Xu H**, Zou S, Xu X. and Zhang L. (2016). Anti-tumor effect of β -glucan from *Lentinus edodes* and the underlying mechanism. *Scientific Reports*. 6:28802. doi:10.1038/srep28802.
- **Yamamoto A.**, Kimura T., Sugitachi A., and Matsuura N. (2009). Anti-angiogenic and Anti-metastatic Effects of b-1,3-D-Glucan Purified from Hanabiratake, *Sparassis crispa*. *Biol. Pharm. Bull.* 32(2) 259—263.
- **Yan J.**; Allendorf, DJ. and Brandley, B. (2005). Yeast whole glucan particle (WGP) b-glucan in conjunction with anti tumour.
- **Yang Z**, Xu J, Fu Q, Fu X, and Shu T. (2013) Antitumor activity of a polysaccharide from *Pleurotus eryngii* on mice bearing renal cancer. *Carbohydr Polym* 95: 615-620.
- **Yeun-hwa Gu**, Yasuyuki Takagi, Takashi Nakamura, Takeo Hasegawa, Ikukatsu Suzuki, Masami Oshima, Hitoshi Tawaraya, and Yoshimi Niwano. (2005). *Journal of Medicinal Food*. 8(2): 154-158. doi:10.1089/jmf.2005.8.154.
- **Yoon, T.J.**, Kim, T.J., Lee, H., Shin, K.S., Yun, Y.P., Moon, W.K., Kim, D.W. and Lee, K.H. (2008). Antitumor metastatic activity of beta-glucan purified from mutated *Saccharomyces cerevisiae*. *International Immunopharmacology* 8(1): 36-42.
- **Zeković**, and Djordje B. (2008). "Natural and Modified (1→3)- β -D-Glucans in Health Promotion and Disease Alleviation". *Critical Reviews in Biotechnology*.

- **Zervakis**, Georgios I.; Venturella, Giuseppe; Papadopoulou, Kalliopi (2001). "Genetic polymorphism and taxonomic infrastructure of the *Pleurotus eryngii* species-complex as determined by RAPD analysis, isozyme profiles and ecomorphological characters". *Microbiology*. 147 (11): 3183–3194. doi:10.1099/00221287-147-11-3183.
- **Zhang Y**, Li S, and Wang X. (2011) Advances in lentinan: Isolation, structure, chain conformation and bioactivities. *Food Hydrocol*. 25:196-206.
- **Zhang, M.**; Chiu, L. C.; Cheung, P. C. and Ooi, V. E. (2006). Growth inhibitory effects of a beta-glucan from the mycelium of *Poria cocos* on human breast carcinoma MCF-7 cells: cell cycle arrest and apoptosis induction. *Oncol.Rep.*15 (3): 637-643.
- **Ziche M.**, Donnini S., and Morbidelli L. (2004) Development of new drugs in angiogenesis. *Curr. Drug Targets* 5, 485–493.

الملخص

اجريت هذه الدراسة لتقييم تأثير البييتاكلوكان المستخلص من الفطر *Pleurotus eryngii* كمادة مضادة للاورام، وتكوين الاوعية الدموية. تم الحصول على عينات الفطر المجفف من وزارة الزراعة\قسم الزراعة العضوية في بغداد. استخلص البييتاكلوكان بأستخدام الماء الساخن ودرس التركيب الكيميائي بأستخدام الـ (HPLC) و الـ (FT-IR) وحدد المحتوى البروتيني والكاربوهيدراتي لعينات البييتاكلوكان المستخلص و اشارت النتائج الى ان المحتوى الكاربوهيدراتي كان (250 mg/ml) والمحتوى البروتيني كان (0.04 mg/ml) على التوالي.

درس التأثير المضاد للعائية بأستخدام طريقة CAM في البيوض المخصبة بعد مرور ٨ ايام على التلقيح واستخدمت جرعة مختلفة من البييتاكلوكان (250,500,1000 and 2000 mg/egg) و اظهرت النتائج انخفاضاً معنوياً في عدد الاوعية الدموية في البيوض المخصبة بالمقارنة مع البيوض القياسية السلبية. لم الجرعة ٢٥٠ mg/Kg انخفاضاً معنوياً في عدد الاوعية الدموية في البيوض المخصبة بالمقارنة مع البيوض القياسية السلبية.

قيم التأثير المضاد للورم في الفئران الحاملة لخط خلايا AN3 adenocarcinoma بعد حقن هذه الخلايا تحت الجلد وتمت دراسة تأثير البييتاكلوكان المعالج على الفئران بعد حقنها به ٥ ملم من الجرعة المختلفة (200, 400 and 800 mg/kg\Day) داخل منطقة الصفاق ولمدة خمسة ايام، اظهرت نتائج الدراسة النسيجية وجود نخر في الورم مع زيادة في عدد الخلايا المناعية بالمقارنة مع الاورام القياسية السلبية.

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

اللَّهُ نُورُ السَّمَاوَاتِ وَالْأَرْضِ مِثْلُ نُورِهِ كَمِشْكَاةٍ فِيهَا مِصْبَاحٌ الْمِصْبَاحُ فِي رُجَاةٍ
الرُّجَاةُ كَانَتْهَا كَوْكَبٌ كَارٍ يُوْقَدُ مِنْ شَجَرَةٍ مُبَارَكَةٍ زَيْتُونَةٍ لَا شَرْقِيَّةٍ وَلَا غَرْبِيَّةٍ
يَمُكِّنُ زَيْتُهَا يُضِيءُ وَلَوْ لَمْ تَمْسَسْهُ نَارٌ
نُورٌ عَلَى نُورٍ يَهْدِي اللَّهُ لِنُورِهِ مَنْ يَشَاءُ وَيَضْرِبُ اللَّهُ الْأَمْثَالَ لِلنَّاسِ وَاللَّهُ بِكُلِّ شَيْءٍ عَلِيمٌ

{سورة النور: الآية ٣٥}



جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقنية الاحيائية

تقييم تأثير البيتا كلوكان المستخلص من *P. eryngii* كمضاد لتكوين الاوعية الدموية

رسالة

مقدمة الى مجلس كلية العلوم جامعة النهرين

كجزء من متطلبات نيل درجة الماجستير في علوم التقنية الاحيائية

من قبل

نور صباح سالم

بكلوريوس تقنية احيائية-كلية العلوم-جامعة النهرين-٢٠١٤

بأشراف

أ.م.د شهلاء مهدي صالح

ايار ٢٠١٧

شوال ١٤٣٨