Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science
Department of Biotechnology

Effect of β-Glucan Extracted from *Saccharomyces cerevisiae* on angiogenesis

A Thesis

Submitted to the College Board of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of the Master of the Science in Biotechnology

By

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March 2014

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ACKNOWLEDGEMENTS

First of all praise is to Allah the first cause of all causes and the glorious creator of the world, mercy and pace to Prophet Mohammed and His Relatives and Companions.

*I would like to express my grateful thank to my super visors, Dr.Shatha Salman and Dr. Ali Z. Al-Saffar*for *encouraging me to continue my studies and formtheir unweaving support during the period of my study.*

I wish also to acknowledge the Head and the of Biotechnology Department in Al-NahrainUniversity, Dr. HamedMajeed and the staff for their appreciable help especially my friends Oroba, Tania, , Dr. Asmma ,Dr.Shyma'a and Dr. Rehab.

I have to express my special thanks and gratitude to the staff of the laboratory analysis of Livestock department /Agricultural research service especially Mr. Nabeel Adel Kamal, Dr. Ammar M. Al-Zubaeede and Zainab A. Al-Abassfor their assistance and kind advice.

Words of thanks are due to my family,especially to soul of my father that support me every moment, I'm so proud to see his name complete my name, great thanks to my mother that help me in my study and great thanks to my husband Ahmed the best friend, for hem steadfast love, patience, supporting and understanding, and thanks to my brothers and sister who shared with me the happens and support me in my study.

Hiba

SUMMARY:

This study was conducted for investigating the effect of β-glucan as anti-angiogenicagent and their immunological effect. Sample of dried *Saccharomycescerevisiae* were obtained from local market in Iraq, the identification has been confirmed the type of *S. cerevisiae*by cultural, morphological and biochemical tests for the yeast.

Glucan was extracted from yeast sample depended on alkaline-acidic hydrolysis method, the net dry weight of glucan was 8.8g / 100g of the yeast.

Carbohydrates and proteins contents were determined for the glucan sample andresults indicated that the percentage of carbohydrates and proteins of glucan were 44% and0.45% respectively.The glucan was analyzed by the FT-IR and result confirmed that the extracted glucan showed high degree of similarity and purity as compared with the standard and the extracted glucan was considered asβ-glucan with the absences of other carbohydrate compound like mannan and glycogen.On the other hand HPLC analysis indicated thatthe extracted β- glucan had the same retention time as compared with the standard β-glucan.

The molecular weight of glucan was estimated by gel filtration chromatography usingSephacryl S-300 column.The detected molecular weight of the β-glucan was300 KDa.

Evaluating the *in vivo*anti-angiogenic effect of β-glucanby CAM assay was carried out using fertilized eggs with age of 8 days.Different concentrations (250, 500, 750, 1000 and 1500 μg/egg) of β-glucan were used and in comparison with the negative control.Results showedthat the

concentrations500, 750, 1000, 1500µg/ml had significant(P≤0.05) effects on the neovascularization that causedinhibitionof the fertilized eggangiogenesis, while the concentration 250µg/mlshowed no significant(P≤0.05) effect of the neovascularization.

Theeffect of phagocytic function of blood phagocytic cells toward yeast cell in the presence of β-glucan was determined*in vitro*.Different concentrations (250, 500,750, 1000 and 1500µg/ml) of the β-glucan sample were used. Theconcentrations 500, 750, 1000 and 1500µg/mlhad a significant(P≤0.05) effect on increasing the phagocytosis function, butthe concentration 1500µg/ml was the most effective in increasing the phagocytosis rate to70%, while the concentration 250µm/ml had no significant effect on the phagocytosis assay.

The mouse serum level of VEGF was evaluated after the intraperitoneal injection of the different concentration (10, 20,30,40,60 mg/Kg)of the β-glucanwas performed.It was found that20, 30, 40 and60mg/Kg had significant effectsin decreasing the level of the mouse VEGF, while 10mg/Kgshowed no effect on the mouse VEGF.

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LIST OF ABBREVIATIONS

VEGF	**Vascular endothelial growth factor**
IL	**Interleukins**
Th	**T- helper cell**
DC	**dendritic cells**
WGP	**Yeast whole β-glucan particles**
PGG	**Betafactin glucan granules**
SSG	**Scleroglucan**
CR	**Complement receptors**
FGF	**Fibroblast growth factor**
MMp	**Matrix Metalloproteinase**
DII4	**Delta-like Ligand 4**
CAM	**Chorioallantoic Membrane**
YNB	**Yeast nitrogen base**
BSA	**Bovine Serum Albumin**
PBS	**Phosphate buffer Saline**
YEPD	**Yeast Extract Peptone Dextrose Broth**
FTIR	**Fourier Transformed Infrared**
V0	**Void Volume**
Ve	**Elution Volume**
HPLC	**High Performance Liquid Chromatography**
TNF	**Tumor necrosis factor**
PMNc	**polymorphic nuclear cells**
ECs	**Endothelial cells**

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V0	**Void Volume**
Ve	**Elution Volume**
WGP	**Yeast whole β-glucan particles**
YEPD	**Yeast Extract Peptone Dextrose Broth**
YNB	**Yeast nitrogen base**

Chapter one
Introduction and literature review

1. INTRODUCTION AND LITERATURES REVIEWE

1.1 Introduction

Throughout the centuries natural compounds have beenwidely used for the prevention and treatment of different diseases. One of the groupsof natural compounds that has attracted increased interest of researchers and clinicians are fungal polysaccharides (Wasser, 2002). Along with the macroscopic fungi, or mushrooms, yeasts also represent a source of valuable polysaccharides contained in their cell walls.

The yeast cell wall is a thick envelope (100 to 200 nm)representing 15–25% of the dry mass of the cell. The major components of cell wall are polysaccharides (up to 90%), mainly β-D-glucans and α-D-mannans with a minor amount of chitin that constitutes only about 1–2% of the polysaccharides and is located predominantly in the bud scars. Glucansrepresenting 50-60% of all cell wall polysaccharides are principal structural components that play role of a skeletal carcass defining rigidity and stability of the cell and its morphological shape (Cabib, 1991; Kogan*et al*., 2008).

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 andSeviour, 2007).

Basically, those studies revealed that beta-glucan is clarified for its propertyto enhance immune system. In addition, beta -glucan, shows strong efficacy for activating macrophages and neutrophils. Beta-glucan-activated macrophages or neutrophils can recognize and kill abnormal cells including abnormal cancerous cells (Pelley and Strickland, 2000; MayellandMaitake, 2001).

Besides to the immunological effects of beta-glucan, many studies proved that Beta-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, 2007), and finally anti-angiogenic properties (Yamamoto*et al*., 2009).

According to the information mentioned above, this study aims to studysome biological properties of Beta-glucan extracted from *Sacchromysiscerevisiae* through:

- Chemical characterization of extracted Beta-glucan.
- Determination the ability ofBeta-glucan to induce phagocytosis.
- Revealing the activity of extracted Beta-glucan in angiogenesis.
- Detecting the effect of Beta-glucan on mouse vascular endothelial growth factor (VEGF) including*in vivo* administration and *in vitro* detection.

1.2 Literatures Review

1.2.1 Glucans

Glucan is a polysaccharide of D-glucose monomers linked by glycosidic bonds. A few bacteria and yeasts employ large extracellular glucosyltransferase for the synthesis of high molecular mass glucans from sucrose (Kuramitsu, 1975; Freimunda*et al*.,2003; Kim *et al*., 2003).Although high similarity exists between these glucansucrase enzymes, they are able to synthesize glucans with different types of glucosidic linkages.

Glucans can be divided into the following two groups:

- α-Glucans, which contains large amounts of glycosidic bonds of the alpha form. Dextran is typical α-glucan which consists of α-1,6glycosidic 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*etal*., 2009).
- β-Glucans, naturally occurring polysaccharides with poly-branched 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*etal*., 2007; Sener*et al*., 2007).

Oat and barley β-glucans are primarily linear with large regions of β(1,4) linkages separating shorter stretches of β(1,3) structures. Mushrooms β glucans have short β(1,6)-linked branches coming off of the β(1,3) backbone. Yeast β-glucans have β(1,6) branches that are further elaborated with additional β(1,3) regions(Dalia*et al*., 2007). Include the glucans have notably increased in the last few years with the exploitation of their biological activities.The term beta-(1, 3)-D-glucans includes a very large number of polysaccharides from bacterial, fungal

and vegetable sources. Their structures have a common backbone of beta-(1,3) linked glucopyranosyl residues but the polysaccharidic chain can be beta-(1,6) branched with glucose or integrate some beta-(1,4) linked glucopyranosyl residues in the main chain (Laroch and Michaud, 2007).

1.2.2 Types of β-Glucans

1- Linear β-(1,3)-D-Glucans (Curdlan)

Curdlanis a neutral gel-forming b-(1,3)-D-glucan product as exopolysaccharide from some bacteria species .Curdlan polysaccharide consist of as many as 12,000 glucose units (Fig1-1) and is insoluble in water, alcohols and most organic solvents, but dissolve in dilute bases (0.25 M NaOH), dimethylsulfoxide (DMSO) and formic acid (AL-Rubaee, 2008).

Curdlan forms a weak gel on heating above 55°C followed by cooling. Further heating to 80-100°C increases the gel strength and produces a firm, resilient gel, while autoclaving at 120°C converts the molecular structure to a triple helix. The gel formed by this high-temperature treatment no longer melts when heated (Laroch and Michaud, 2007).

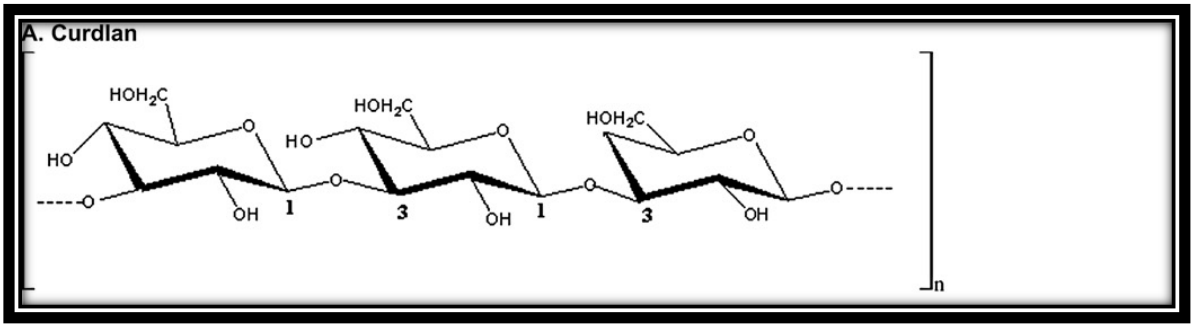


Fig (1-1): Chemical structure of linearβ-(1,3)-D-Glucans (Laroch and Michaud, 2007)

2- β-(1,3) (1,4)-D-Glucans

It is one of the important compound in the cell wall of the wheat and barley and it concentrated in the cell wall of plant seed (endosperm) and also in the aleuronic layer for the wheat ,oat and white corn (AL-Rubaee, 2008) the wheat and oat contain large amount of β-*(*1-3) (1-4) glucan about 2-14% of the dry weight of the cells and viscoelastic characteristics of β-glucan gels are related to the molecular weight of the isolated fractions. Differences in molecular weight were observed between barley and oat β-glucans, and among β- glucans extracted from different cultivars of barley (Laroch and Michaud, 2007).

3- 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, 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 back bone 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(1-6)(1-3) –D-glucan can be extracted from different microorganisms, however, it 'is very important to consider that β-glucan in *Sacchromysiscerevisiae*contribute about 55-65%of the cell wall and have many important physical and biological activities for many medical and industrial applications (Klis*et al*.,2002; Thammakiti*etal*., 2004).

The beta (1,3) and (1,6) glucan produced from different organism (table1.1) and *Saccharomyces cerevisiae*is the important source of the glucan.

Table(1-1):Types and sources of Glucan

Organism produce glucan	Common Name of Glucan	Branches	Molecular Weight	references
Agrobacterium sp	Curdlan	Liner β*(1,3*)	$3.5x10^{4}2x10^{6}$	(Lee, 2001)
Grifolafrondosa	Grifolan	Branch β*(1,3)*	$4.5x10^{5}$	(Kato *et al.*,1983)
Lentinuseeodes	Lentinan	*B(1,3)*branched by β*(1,6)*	$5x10^{5}$	(Saito *et al.*,1979)
Schyzophyllumcommun	Schyzophyllan	One branch for each 3glucose	$4.5x10^{5}$	(Saito *et al.*,1979)
Sclerotiumglucanum	Sclerotoglucun	One branch for each 3glucose	$1.6x10^{6}$-$5x10^{6}$	(Lee, 2001)
Sclerotinia sclrtotiorum	SSG	High branch of β*(1,3)*	$2x10^{5}$-$2x10^{6}$	(Lee, 2001)
Pariacocos	Pachyman	Same of branches in β*(1,6)*	$2,06x10^{4}$, $8.93x10^{4}$	(Saito *et al.*,1979)
Coiolusversicolor	Krestin	Non branched *β(1,3)*	$1.0x10^{6}$	(Lee, 2001)
Sacchromysiscerevisiae	Neutral soluble glucan (NSG)	β*(1,3)*branched by *B(1,6)* bond	$2x10^{5}$-$2x10^{6}$	(Williams *et al.*, 1994)
	PPG glucan	β*(1,3)*branched by *B(1,6)* bond	$2x10^{4}$-$15x10^{4}$	(Li *et al.*, 2006)
	Particulate Glucan insoluble	β*(1,3)*branched by *B(1,6)* bond	$3.53x10^{4}$-$4.75x10^{6}$	(Williams *et al.*, 1994)
Oat	Oat Glucan	Liner nonbranchedβ*(1,3) (1,4*	0.65-$3x10^{6}$	(Beer *et al.*,1997)

Barley	Barley Glucan	Linear non-branched β(1,3)(1.4)	$0.15x10^6$-$2.5x10^6$	(Beer *et al.*,1997)
Laminaria digitala	Laminarian	*β(1,3)B(1,6)*	---------	(Mullere *et al*., 2000)

1.2.3*Saccharomyces cerevisia*

Saccharomyces cerevisiae a yeast that has been used in the fermentation of food and drink for thousands ofyears and is traditionally considered as safe, with GRAS (generally regarded as safe) status in the food industry(Mackenzie, *et al*., 2008).

Saccharomyces cerevisiae is classified according to Herskowitz (1988) as follow:

Kingdom:	Fungi
Phylum:	Ascomycota
Subphylum:	Saccharomycotina
Class:	Saccharomycetes
Order:	Saccharomycetales
Family:	Saccharomycetaceae
Genus:	*Saccharomyces*
Species:	***cerevisiae***

*Saccharomyces cerevisiae*was adopted as a model system for laboratory study in the 1930s, as investigators developed genetic tools to understand its life cycle and differentiation (Hall and Linder, 1993).It provided an important tool to understand recombination and the transmission of genetic material, and launched into greater prominence with the molecular era in the1970s. It has highly organized internal structures with the membrane-delimited compartments typical of eukaryotic cells, including a nucleus, mitochondria, Golgi and other structures. *S. cerevisiae* grows by budding, which requires highly targeted cell growth and mechanisms for spatial coordination with nuclear division (Pruyne*etal*., 2004).

In *S. cerevisiae*, the cell wall makes up 15–30% of the dry weight and 25–50% of the volume of the cell, which protects it from osmotic pressure and environmental stress, and determines cell shape(Kath andKulicke, 1999). The cell wall of *S. cerevisiae* is organized into two layers which are made up of four classes of macromolecules, namely mannoproteins, β(1, 3)-glucan, β(1,6)-glucan and chitin (Table 1.2). These components are all interconnected by covalent bonds, mannoproteins account for approximately 35–40% of the dry weight of the cell wall. More than half the cell wall (50–55%) is made up of β(1, 3)-glucan (Shokri*etal*., 2008).

In recent years, increasing attention has been paid to β-glucans isolated from the cell wall of yeast. In respect to the high incidence of life-threatening infections among cancer patients, transplant recipients, patients with AIDS and patients receiving broad-spectrum antibiotic, corticosteroid and cytotoxic drugs, widespread efforts have been made to identify immunomodulatory agents (Shokri*et al.*, 2008).

Various methods were used to purify β-glucan from the yeast cell wall during the last decades (Nguyen *et al*., 1998).Numerous studies have demonstrated that *B*-glucans, either soluble or particulate, isolated from the yeast, exhibit antitumor, antimicrobial and radio-protective activities (Shokri*et al.*,2008).

Table (1-2):Cell wall components of *Saccharomyces cerevisiae*(Klis*etal*., 2002; LessageandBussey, 2006; Kwiatkowski *etal*.,2009)

Component	Cell Wall Mass %, Dry Weight
(1→3)-β-D-glucan	50-55
(1→6)-β-D-glucan	5-10
(1→4)-α-(1→3)-β-D-glucan	3-7
Mannoprotein complex	35-40
Chitin	2

The solubility of β-g1ucans is associated with the degree of polymerization (DP)(Mario *et* al., 2007). β-glucans can be classified according to their solubility properties into:

(a) Alkali insoluble, acetic acid insoluble (1 ⟶3)-β-g1ucan.

(b) Alkali-soluble (1 -⟶3)-β-g1ucan.

(c) Highly branched (1 ⟶6)-β-g1ucan.

1.2.4 Effects of β-glucans on the immune system

Some fungal β-glucans markedly stimulating the immune system and induce protection against pathogenic microbes and from harmful

effects of environmental toxins and carcinogens (VetvickaandYvin 2004; Brown and Gordon, 2005).

β-glucans are not synthesized by humans, so these compounds are recognized by our immune systems as non-self-molecules , inducing both innat and adaptive immune responses (Brown and Gordon 2005).

1.2.4.1-Effects of the β-glucan on the innate immune system

Innate immunity is present when we are born, and is a relatively non-specific system, responding against many, but not all, structurally related antigens (Brown and Gordon 2005;Munz*etal*., 2005).

Certain β-glucans, including zymosan, grifolan and lentinan, appear to activate phagocytes, thus leading to elimination of pathogens by phagocytosis (Ladanyi*etal*., 1993; Kurashige*etal*., 1997; Brown *etal*., 2002).Among these, the macrophages preferentially attack dead cells and intracellular pathogens (Munz*et al.*, 2005).

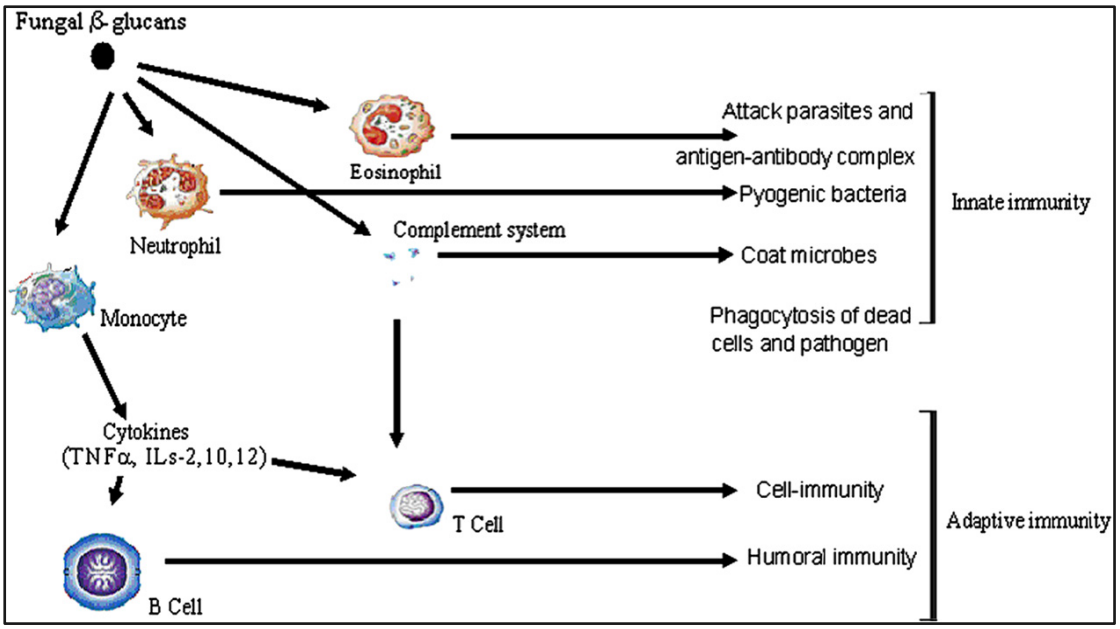
In addition, many reports indicated that β-glucan from *Saccharomyces cerevisia*can stimulate macrophages to produce cytokines like IL-2,IL-10 and IL-12, local immunomodulators, andthese in turn activate adaptive immunity (Brown *et al*., 2002; Sato *et al*., 2003; Young *et al*., 2004).

Cells of the innate immunity have surface beta-glucan receptors, which specifically recognize and bind the β- (1, 3)glucan linkage of the β-glucanmolecule also lymphocytes belong to the acquired immunity play a key role in defending the body against disease (Xiao *et al*., 2004). Bacterial or fungal products can initiate the immune response mostly by binding to the innate immune receptors (Underhill *et al*., 2005).

1.2.4.2-Effects of β-glucan on adaptive immunity

The adaptive immune system responds to introduced foreign antigens. It involves both B and T cells, the former produce antibodies to mediate humoral immunity, whereas T cells induce cell-mediated immunity (Munz*et al*., 2005). Cytokines promote T cell differentiation to helper T cells 1 (Th1) and 2 (Th2), which mediate cell and humoral immunities, respectively (Trinchieri, 2003).The adaptive immune response also involves dendritic cells (DC), derived from monocytes, and these present antigens to T-cells for activation of immune responses (Munz*et al*., 2005). These DC are activated, thus facilitating the expression of adaptive immunity (Kanazawa *et al*., 2004; Munz*et al*,. 2005).

Fungal b-glucan-induced immune responses are different in their actions to immune therapies based on supplementation of elements of the immune system, e.g. exposure to IL2 and interferon gamma. Instead, they appear to act by stimulating the whole immune system (Fig.1.2). Consequently, these b-glucans may have an advantage in treating diseases. Furthermore, many can be administered orally and combinational application with other immune therapies may generate a more potent end result (Berner*etal*., 2005; Drandarska*etal*., 2005).



Fig(1.2):Immunostimulation by fungal β-glucan(Berner *et al*., 2005)

1.2.5 Importance of β-Glucans in the Treatment of Cancer

Cancer is uncontrolled cell proliferation induced by many factors including environmental chemicals, viruses, bacteria, hormones, and chronic inflammation (Nam *et al*., 2005). Three developmental stages are recognized. The first is 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 activation of a tumor promoter that leads to the formation of small benign tumors. Finally in the third stage,progression, the normal tight control over the cell cycle is lost, resulting in uncontrolled cell proliferation (Borchers *etal*., 2004; Nam *etal*., 2005).

Although surgical resection remains the most effective early treatment of solid cancers, chemotherapies and immunotherapies are often used (Nilsson *et al*. 2004). Progress in understanding the molecular

mechanisms of carcinogenesis has allowed these non-surgical therapies to become more effective. Some fungal *β*-glucans appear to beneficially influence both cancer promotion and progression (Takaku*et al.*, 2001; Nilsson *etal*., 2004; Nam *etal*., 2005), and such treatment of rats has led to formation of much smaller tumors than those seen in controls (VetvickaandYvin, 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 neuroblastomatumor regression and long-term survival in mice (Cheung *etal*., 2002; Cheung andModak 2002; Yan *etal*., 2005). In mice with established subcutaneous non- Hodgkin's lymphoma xenografts, a combination of intravenous complement-activatingantibody, rituximab, and WGP from yeast had a higher therapeutic efficacy than treatment with either alone (Modak *etal*., 2005).

1.2.6 Anti-bacterial Activities of β-Glucan

Some β-glucans, including lentinan, WGP, PGG, and SSG, are also effective against bacterial infections. Thus, lentinan reduced *Mycobacterium tuberculosis* infections by increasing macrophage levels in vivo in a rat model and in vitro examinations showed these macrophages had increased killing ability toward *M. tuberculosis*cells (Markova *et al*., 2005). Both PGG and WGP have effectively treated mice against *Bacillus anthrax* infections (Kournikakis*et al*., 2003), correlating with increases in levels of cytokines IL-2 and IL-10, although experiments to clarify their modes of action by blocking cytokines were not conducted.

PGG from *Saccharomyces cerevisiae* also effectively treated *Staphylococcus aureus* infections involving strains resistant to several b lactam antibiotics including methicillin, by improving patient survival by 80 % (Liang *et al*., 1998). It also successfully treated methicillin-resistant strains of *S. aureus* and *S. epidermidis* in guinea pigs (Kernodle*et al*., 1998). As with other examples given here, only a restricted range of β-glucans have been examined in this way and further work with others seems warranted. PGG can also prevent wound infections, and was more effective in combination with the antibiotic cefazolin than alone (Kaiser andKernodle, 1998).

1.2.7 β- Glucan andwound healing

Macrophage activities stimulated by β-glucans from *Saccharomyces cerevisiae*may also benefit wound healing and reduce scar tissue levels after surgery or trauma, as revealed by both animal and human studies (Mayell, 2001; Portera*et al*., 1997). In normal human dermal fibroblasts this β-glucan preparation stimulates procollagen mRNA and collagen biosynthesis (Wei *et al*., 2002).

Inhibition of Neurofibromatosis typ-1(NF-1) by blocked induction of procollagen mRNA by the same β-glucan, which also induced mRNA synthesis of many other wound growth factors including activator protein, specificity protein, neurotrophin, platelet-derived, fibroblast growth factor, and transforming growth factor (Wei *et al*., 2002).

1.2.8 Application of β-Glucans in Food Products

Apart from health and nutritional benefits (Malkki and Virtanen, 2001), β-glucan also has various suitable functional properties such as thickening, stabilizing, emulsification, and gelation. These properties determine the suitability of β-glucan to be incorporated in soups, sauces,

beverages, and in other food products (Dawkins and Nnanna, 1995; Burkus and Temelli, 2000). Barley β-glucan is particularly well suited for such applications, being capable of imparting a smooth mouth feel to beverage products, and also makes the beverage an excellent source of soluble dietary fiber. Its properties enable it to be incorporated alternatively in traditional beverage thickeners as replacement for gum Arabic, alginates, pectin, xanthan gum, and carboxymethyl-cellulose (Giese, 1992). Some studies focused to explore the ways to incorporate β-glucans into various food systems (Hallfrisch and Behall, 1997; Ahmad *et al*., 2008). In this context, β glucan is extracted from different sources and marketed in various forms such as β-glucan concentrate extract from oats "Oattrime", β-glucan from barley "NutrimXe" and β glucan extracted from rice "Ricetrim" (Inglett*et al*., 2004).

1.2.9Antiangiogenic Effect of β-Glucan

β-glucans is likely to involve their specific interaction with several cell surface receptors, as complement receptor 3 (CR3) , lactosylceramide, selected scavenger receptors, and dectin-1 (bGR).Antiangiogenesis can be one of the pathways through which Beta-glucans can reduce tumor proliferation, prevent tumor metastasis (Akramiene*et al*., 2007).

Investigated shows the effect of administration of *Saprasiscrispa*on angiogenesis (it isan edible mushroom) with medicinal properties it contain more than 40% β-glucan researchers found that it can suppress of the primary tumor and extent of the metastatic foci in the lung correlating with inhibition of tumor induced- angiogenesis, also *Coriolusversicolor* mycelia it was found that there was a reaction of tumor weight and

vascular densityvia suppression of vascular endothelial growth factor(VEGF) gene expression (Yamamoto *etal.,* 2009).

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

Quantitative analysis of microcorrosion casting of the tumor tissue showed more angiogenic features such as dense sinusoids and hot spots, in control (untreated) than in treated animals(treated with polysaccharide). Immuno-staining of tumor tissues with antibody against the endothelial cell marker demonstrated a positive correlation in that both the vascular density and tumor weight which is lower in mice treated with polysaccharide. The total amount of new vessels production was reduced; the basic tumor type-specific vascular architecture was retained. However, the expression of vascular endothelial cell growth factor (VEGF) in these tumors was suppressed. So, anti-angiogenesis can be one of the pathways through which β-glucans mediate anticarcinogenic activity (Dalia *et al*., 2007).

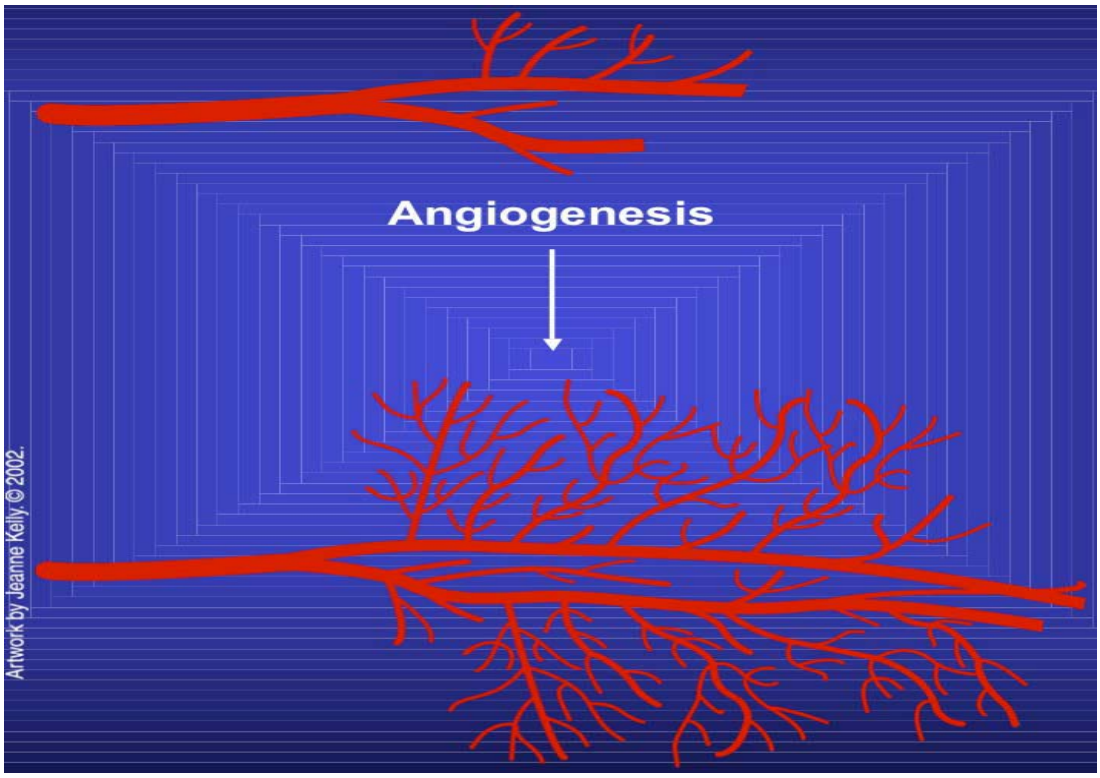
1.2.10 Angiogenesis

Is a multistep process, leading to the formation of new blood vessels from the existing ones. It occurs during the embryonic development, endometrial regulation, the reproductive cycle, and wound healing.Angiogenesis also plays a critical role in many disease conditions like solid tumor progression, metastasis, diabetic retinopathy, arthritis, psoriasis, hemangiomas, and atherosclerosis (Folkman,1995).

Antiangiogenisis is inhibition of blood vessel growth(fig.1.3). 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).

Vessels consist of two main cell types: endothelial cells and mural cells. In adults, formation and growth of new vessels are under strict control. These processes are activated only under strictly defined conditions like wound healing. Strict regulation of this system and balanced functioning is very important for the organism, because both excessive formation of blood vessels and their insufficient development lead to serious diseases (Karamysheva, 2008).

Activation of angiogenesis is necessary condition for tumor development. An expanding tumor nodule, like any other tissue, must be supplied with oxygen and nutrients to maintain its vital activity. It is known that without blood supply the dimensions of tumor nodule cannot exceed 2-3mm3 due to hypoxia leading to death of tumor cell (Folkman, 1995).



Fig(1.3): The process of angiogenesis

1.2.11 Chemical stimulators of angiogenesis

Chemical stimulation of angiogenesis is performed by various angiogenic proteins, including several growth factors:

1) **FibroblastGrowth Factor (FGF)**

The fibroblast growth factor (FGF) family with its prototype members FGF-1,(acidic FGF) and FGF-2 (basic FGF) consists to date of at least 22 members, most are single chain peptides of 16-18 KDa and display high affinity to heparin and heparin sulfate. In general, FGFs stimulate a variety of cellular functions by binding to cell surface FGF-receptors in the presence of heparin proteoglycans (Ornitz and Itoch, 2001).

FGF-1 stimulates the proliferation and differentiation of all cell types necessary for building an arterial vessel, including endothelial cells and smooth muscle cells; this fact distinguishesFGF-1 from other pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF), which primarily drives the formation of new capillaries (Khurana and Simons, 2003).

2) Angiopoietins

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

3) Matrix Metalloproteinase(MMP)

Another major contributor to angiogenesis is matrix metalloproteinase (MMP). MMPs help degrade the proteins that keep the vessel walls solid. This proteolysis allows the endothelial cells to escape into the interstitial matrix as seen in sprouting angiogenesis. Inhibition of MMPs prevents the formation of new capillaries (Haas *et al*., 2000).These enzymes are highly regulated during the vessel formation process because destruction of the extracellular matrix would decrease the integrity of the microvasculature (Prior*et al*., 2004).

4) Delta-like Ligand 4 (DII4)

Delta-like ligand 4 (DII4) is a recently discovered protein with an important negative regulatory effect on angiogenesis (Lobov*et al*., 2007).

5) Vascular Endothelial Growth Factor (VEGF)

VEGF has been demonstrated to be a major contributor to angiogenesis, increasing the number of capillaries in a given network. Initial *in vitro* studies demonstrated bovine capillary endothelial cells will proliferate and show signs of tube structures upon stimulation by VEGF and FGF2, although the results were more pronounced with VEGF (Goto*et al*,.1993*).* Up regulation of VEGF is a major component of the physiological response to exercise and its role in angiogenesis is suspected to be a possible treatment in vascular injuries (Ding YH *etal*,. 2004; Gavin *et al*., 2004; Kraus *etal*.,2004).

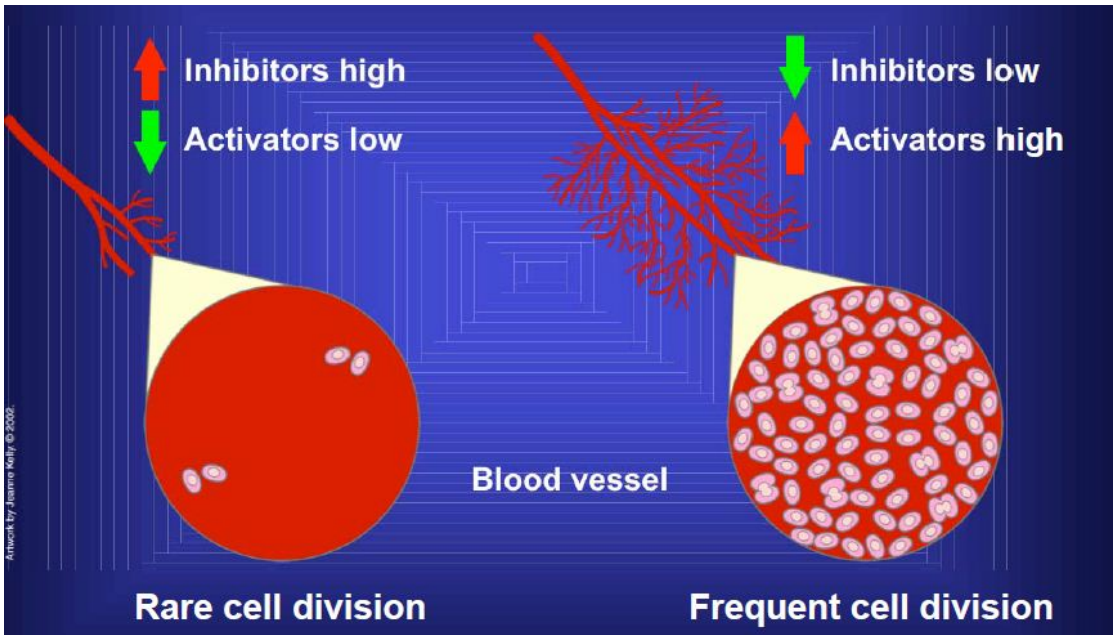
In vitro studies clearly demonstrate that VEGF is a potent stimulator of angiogenesis because, in the presence of this growth factor, plated endothelial cells will proliferate and migrate, eventually forming tube structures resembling capillaries (Fig 1.4) (Prior *et al*., 2004).VEGF is also known as a factor regulating vascular permeability and the ability of this factor to enhance vascular permeability defines its important role in inflammation and other pathological processes .In particular, it is known that tumor vessels are characterized by enhanced permeability, and this peculiarity contributes to tumor cell penetration into vascular networks and metastasis (Karamysheva, 2008).

1.2.12 Angiogenesis Inhibitors

More than 300 angiogenesis inhibitors have been discovered so far, and more than 80 drugs derived from them are in clinical trials (Madhusudan and Harris,2002). Though these angiogenesis inhibitors are effective against a variety of tumors, high dosages of these substances are

necessary to suppress tumor growth. Other disadvantages of antiangiogenic protein therapy include the need for repeated injections and prolonged treatment, the transmission of toxins and infectious particles, and the high manufacturing cost (Gastl et *al.*, 1997). In these context natural resources for the exploration of antiangiogenic substances hold great promise. A variety of antiangiogenic substances have also been isolated from natural sources, including shark cartilage, curcumin, the x-3 and x-6 fatty acids, green tea, licorice, quercetin, squalamine, and vitamin D3 (Marwick, 2001). Apart from shark cartilage (Cho and Kim, 2002; Gingras*etal*., 2003), some marine natural compounds from sponges (Zhou *etal*., 2000; Fujita *etal*., 2001; Shin *etal*., 2001; Rodriguez-Nieto

etal., 2002; Williams *et al*., 2002) and sponge-associated bacteria (Muller *et al*., 2004) have also been reported to possess antiangiogenic potential also the antiangiogenic factors isolated from *saccharomyces cerevisia* (Seung*et al*., 2006).



Fig(1.4):Angiogenesis regulated by activators and inhibitors

1.2.13 Study of Anti-angiogenesis by Chick Embryo Chorioallantoic Membrane Assay(CAM)

The classical assays for studying angiogenesis *in vivo* include the rabbit ear chamber, the mouse dorsal skin and air sac, the chick embryochorioallantoic 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 shell-less culture in Petri dishes and plastic wrap/cup apparatus. There is no clear evidence that there is any significant difference between data derived using *in vivo*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 (Ribatti, 2010).

Chapter two
Materials and methods

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Equipment and Apparatuses

The following equipment and apparatus were used during this study:

Equipment	Company (Origin)
Autoclave	Express (Germany)
Balance	Ohans (France)
Centrifuge	Hermle Z200A (Germany)
Compound Light Microscope	Olympus (Japan)
Cooling centrifuge	Harrier (U.K)
Eppendorf bench centrifuge	Netherlerand Hinz (Germany)
Haemocytometer	Neubaur (Germany)
HPLC, FTIR	Shimadzu (Japan)
Incubator	Termaks (U.K)
Laminar air flow hood	Heraeus (Germany)
Micropipette	Witey (Germany)
Microtiter Plate Reader	Bio-Rad (Germany)
Oven	Gallen Kamp Sayo(U.K)
pH-meter	Metler Toledo (U.K)
Sensitive Balance	Metler AE 260(Switzerland)
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, Dextran blue - 2000.	LKB (Sweden)
Fructose, Agar, Sucrose, Maltose, Galactose, Lactose, Tris-HCl, Potassium bromide	Fluka (Germany)
Glucose, Phosphoric acid, Ethanol absolute, EDTA, Dextrose, Acetone, NaOH, KCl, NaCl.	BDH (England)
Heparin.	Panapharma (France)
Peptone	Bio-life (Italy)
Phenol, Urea.	Sigma (Germany)
Potassium dihydrogen phosphate, Absolut methanol, Glacial acetic acid, NaH_2PO_4.	Analar (England)
Rafinose, Urea agar base	Difco (USA)
Sephacryl-300.	Pharmacia (Sweden)
Yeast extract, Yeast nitrogen base	Oxiod (England)

2.1.3 Stains

Stains	Company (Origin)
MethylenBlue	Fluka (Germany)
Giemsastain	BDH (England)

2.1.4Kits

Kits	Company (Origin)
Mouse vascular endothelial growth factor Kit	R&D System (USA)

2.1.5Standards

Standard	Company (Origin)
Beta-1,3-glucan from *Euglena gracilis*	Sigma (Germany)

2.1.6 Laboratory prepared media

A. Yeast Extract PeptoneDextrose Broth (YEPD)(One*et al*., 1991)

The medium is composed of the following:

Glucose	20g
Peptone	20g
Yeast Extract	10g

The components were dissolvedin 1L distilled water; pH was adjusted to 5.0and sterilized by autoclaving for 15-20min. For YEPD solid medium preparation, 15-20g/L agar was added.

B. SugarFermentationMedium(Lodder, 1974)

The medium is composed of the following:

Yeast Extract	1g
Peptone	1g
Phenol red	0.0012g

The components were dissolvedin 100mldistilled water and aliquots (4ml) were distributed in test tubes containing small Durham tube upside down and sterilized by autoclaving 4ml of this medium was distributed in each tubes containing (Durham's tube) and sterilized by autoclaving. After cooling, 1ml of different sugar solution (2.1.7.1B), sterilized by filtration was added.

C. SugarAssimilation Medium(Barentt *et al*., 1990)

This medium was prepared by dissolving 6.7g of yeast nitrogen base(YNB) in 100ml of distilled water then 5gof carbon source included either: glucose, fructose, sucrose, lactose, ormaltose was added (except for rafinose, 10g was added). ThepH was adjusted to 5.0 and sterilized by filtering. Aliquots of 0.5mlwere distributed in sterile testtubes and the volume was completed to 5ml using sterile distilled water.

D. Urea Agar Medium (Atlas *et al.*, 1995)

It was prepared according to the recommended instruction by the manufacturing company. The pH of 95ml urea agar base was adjusted to 7.2 and sterilized by autoclaving. After cooling to 50°C, 5ml of 40% urea solution (sterilized by filtration) was added, mixed and distributed into sterilized test tubes in slant position.

2.1.7Preparation of Reagents, Buffers and Dyes

2.1.7.1 Reagents and Dyes Used in*Saccharomyces cerevisae*Identification

A. Methylene Blue Stain (Atlas*et al*., 1995)

This stain was prepared by dissolving 0.3g of methylene blue in 100ml of distilled water then filtered byWhattman No.1 filter paper.

B. Solution for the SugarFermentation Medium(Lodder, 1974)

It was prepared by dissolving 0.5g of sugar (glucose, lactose, sucrose, maltose, fructose and galactose) in 100ml distilled water. The solutions were sterilized by filtration.

2.1.7.2 Solutions for the Determination of Carbohydrate Concentration (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. H_2SO_4 Solution (98%).

2.1.7.3 Solutions Used in Determination of Protein Concentration(Bradford, 1976)

A. Coomassie Brilliant Blue G-250 Stain

It was prepared by dissolving 0.1g of CoomassieBrilliant Blue-G-250 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.

2.1.7.4 Solution and Buffers Used for Glucan Extraction(Byron, 1993)

a. NaOH (1.5 M)

It was prepared by dissolving 60g of NaOH in 1L of distilled water.

b.NaOH (3%)

It was prepared by dissolving 3g of NaOH in 100ml distilled water.

c. Glacial acetic acid (3%)

It was prepared by mixing 97ml distilled water with 3ml glacial acetic acid.

2.1.7.5 Solution and Buffers Used forMolecular WeightDetermination(Rimsten*et al*., 2003)

a. Phosphate buffer Saline(PBS)

This buffer was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.15g $NaH_2PO_4$and 0.2g Na_2HPO_4 in 1L distilled water. The pH was adjusted to 7.2. The solution was sterilized by autoclaving and stored at 4°C until use.

b.Glucan Stock Solution

Glucan stock solution was prepared by dissolving 0.05g of glucan in 1ml of 0.1 M NaOH. The volume was completed to 10ml with PBSandfiltered by Whattman No.1 paper.

c. Standard ProteinsSolution

Standard proteins that used for molecular weight determination were:

StandardProteins	Molecular Weight (Dalton)
Hemoglobin	64,000
pepsin	36,000
urease	480,000
albumin	67,000

A weigh of 0.5g of each of the above proteinswasseparately dissolved in 10ml of PBS to give final concentration of 50mg/ml.

d. Blue dextran solution

This solution was prepared by dissolving 0.05g of blue dextran in 10ml of PBS.

2.1.7.6 Solutions and Dyes Used forPhagocytosis Assay(Furth *et al*., 1985)

a. Yeast suspension

Pureyeast culture of *Saccharomyces cerevisae*was harvested with sterile PBS.Yeast suspension was prepared to the contain$1x10^6$ cell/ml and stored in -20C.

b. Glucan stock solution

Glucan stock solution was prepared by dissolving 0.01 g of glucan in 10ml PBS and filtered byfilter paper (0,45mm).

C. GiemsaStain

This stain was prepared by dissolving 2g of Giemsastain powder in 100ml methanol (free water).Then the solution was heated for 2hr at 50°C with stirring,then incubate the stain at37°C for 24hr. Finally the stain was filtered by using filter paper (Whattman No.1).

D. Sorenson buffer

The buffer was prepared by dissolving 9.47g of Na_2HPO_4 and 9.8g of KH_2PO_4 in 1000ml of distilled water.

2.2. Methods

2.2.1 Sterilization Methods (Atlas *et al*., 1995)

A. Moist Heat Sterilization

All media, buffers and solutions were sterilized by autoclave at 121ºC (15Ib/in²) for15 min, unless otherwise stated.

B. Dry heat sterilization

Electric oven was used to sterilize glassware and others by heating at 180ºC for 2 hours.

C.Filtration (membrane sterilization)

Solutions that sensitive to heat were sterilized by filtration using millipore filters 0.45µm diameter.

2.2.2 Sample Collection of Yeast

Samples of dried bakery yeast(Sef-instant-Francs) were collected from different local markets in Baghdad. The yeast was activated by suspending 0.1g of thesample in 10ml of distilled water,mixed and incubated at 30ºCfor 30 min.

The suspensions were mixed by vortex and cultured by streaking on YEPD agar plates(2.1.6. A), and incubatedat30ºC for 48hr. Single colonies were picked and streaked on YEPD agar plates. Plates were incubated at 30ºC for 48hr.

2.2.3Maintenance of the yeast samples (Maniatis*et al.*, 1982)

A- Short- Term Storage

Yeast isolates were maintained for few weeks on YEPD agar plates; theplates were wrapped tightly with parafilm, and then stored at 4ºC.

B- Medium-Term Storage

Yeast isolates were maintained for few months by stabbing the isolates inscrew-capped YEPD agar slant tubes containing 5-8 ml ofYEPD agar and stored at 4ºC.

2.2.4 Identification of *Saccharomyces cerevisiae*

2.2.4.1Morphological Characteristics (Lodder, 1974)

A. Cultural Characteristics

Cultural characteristics for the *S.cerevisiae*were stated by culturing the yeast on YEPDA and incubated at30ºC for 48hr. The morphological characteristics including colony shape, color and texture were recorded.

B. Microscopic Exam

Athin film of yeast was prepared by smearing a loopful of yeast suspension on the clean glass slide dried and stained with methylene blue, then examined under light microscope (40 X).

2.2.4.2 Biochemical Tests

1) **SugarFermentation (Lodder, 1974)**

Sugar fermentation medium (2.1.6 B) was inoculated with fresh culture of yeast isolate and incubated at 30°C for 48 hours. Changing the color from red to yellow and gas formation indicates a positive result.

2) **SugarAssimilation (Barentt*etal*., 1990)**

Sugar assimilation medium (2.1.6C) was inoculated with fresh culture of yeast isolate and incubated at 30°C for 48 hours. Increase the turbidity of the mediumindicates a positive result.

3) **Urea Hydrolysis Test (Barentt*et al*., 1990)**

This medium (2.1.6.D) was inoculated with fresh culture of yeast isolate and incubated at 30°C for 24hrs. Appearance of growth and changing the color to dark pink means a positive result.

2.2.5Glucan Extraction from*S. cerevisiae*(Byron, 1993)

1. Dried yeast(200g) was mixed with 1L of 1.5 M hot sodium hydroxide(50-60°C).
2. The mixture was autoclaved for 1houre,andthen the mixture was cooled at room temperature.
3. The suspension was centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the pellet was taken.
4. The pellet was washed three times with distilled water and centrifuged at 3000 rpm for 15 min.
5. Thewashed pellet was mixed with1L of 3% glacial acetic acid and heated up to 85°C for 3 hours with stirring.
6. The suspension wascentrifuged at 3000 rpm for 15min. The supernatant was discarded and the pellet was washed with 1L

distilled water. Themixture was centrifuged at 3000 rpm for 15 min and the supernatant was discarded.

7. The pellet was mixed with 600ml of absolute ethanol with stirring.
8. The suspension was centrifuged at 3000 rpm for 15 min and the pellet was separated.
9. The pellet was mixed with 600ml of acetone with stirring then centrifuge the suspension at 3000 rpm for 15 min and the pellet was separated.
10. The pellet was washed with 600ml ethanol (absolute) with stirring.
11. The suspension was centrifugedat 3000 rpm for 15 min and the pellet was dried by oven at 60°C.

2.2.6Analysis of Glucan by FTIR(Fourier Transformed Infrared) (Naja *et al.*, 2005)

The chemical structure of glucan from *S.cerevisiae*was analyzed by Fourier Transformed-Infrared spectrometry (Shimadzu IRAffinity – 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.

2.2.7Determination of Glucan Molecular Weightby Gel Filtration Chromatography.

2.2.7.1 Determination of Void Volume (V0) of theColumn

A Sephacryl S-300 column (2 x 31.5 cm) was used for the detection glucan molecular weight and was equilibrated and washed for 24hr with phosphate buffer saline (2.1.7.5 a)at flow rate of (1ml/min).

Five milliliters of blue dextrane-2000 solution was passed through the columnand eluted with PBS. Fractions of 5ml were collected and absorbency at 600nm for each fraction was measured. The void volume was calculated by determination the number of the fraction in which max absorbance of blue dextran at 600nm was obtained multiplied by the volume of fractions(Rimsten *et al.*, 2003).

2.2.7.2 Determination of Standard Proteins Elution Volume (Ve)

Five milliliters of the standard proteins solutions (hemoglobin, pepsin, urease and albumin) were applied through the column separately, and eluted with PBS buffer at flow rate (1ml/min). The elution volume (Ve) was estimated for each protein after measuring the absorbency of the separated fractions and calculates the volume of the fractions for each proteins peak at 280nm.Ve/Vo ratio was calculated for each protein and standardization was achieved by plotting the (Ve/V0) ratio for each protein versus the log of molecular weight of theprotein. The molecular weight of glucan was calculated depending on the standard curveobtained.

2.2.8Analysis of glucan byHigh Performance Liquid Chromatography(HPLC)Technique:

The samples and standard of B-glucan were analyzed by HPLC separation with column Luna 5u C18 (250x4.6mm) international

diameter. The mobile phase was acetonitrile (CAN) 100% with flow rate of 0.5 ml/min. Injection volume for sample and standard solution was 10µl. The pH was adjusted to 3.5. The detection occurred at UV light at 305 nm wave length.

2.2.9Determination of Protein Concentration

Protein concentration was determined according to Bradford, (1976)as follow:

1.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 (µg/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

2. A volume of 2.5 ml of CoomassieBrilliant Blue G-250 dyewas added to each concentration mixed and left to stand for 2 min at room temperature.

3. 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.

4. A standard curve was plotted between the BSA concentrations against the corresponding absorbance of the bovine serum albumin.

5. Glucan sample (0.1ml of 100µg/mlTris-HCl)was treated as the same previous steps.The protein concentration was calculated from the standard curve (Fig.2-1).

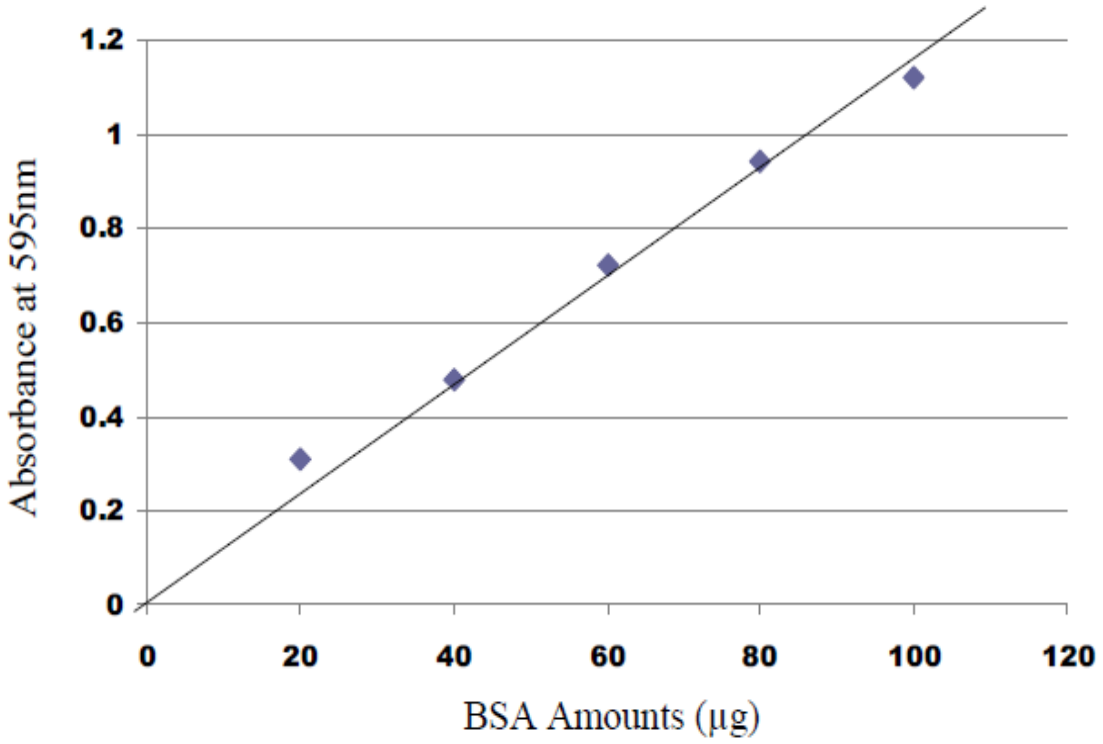


Fig. (2-1): Standard Curve of bovine serum albumin described by method of Bradford, (1976)

2.2.10 Determination of Carbohydrate Concentration

Carbohydrate concentration was determined by phenol - H_2SO_4 method originally described by Dubois *et al*., (1956) as follows:

1. Different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/ml) were prepared from glucose stock solution (2.1.7.2) with final volume of 1 ml. Then 1 ml of phenol solution (5%) was added to each tube with mixing.

2. A volume of 5 ml from H_2SO_4 was added to the mixture with vigorous mixing, and then left to cool at room temperature.

3. 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 H_2SO_4.

4. A standard curve was plotted between the glucose concentrations against the corresponding absorbance (Fig.2-2).

5. 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 (Fig.2-2).

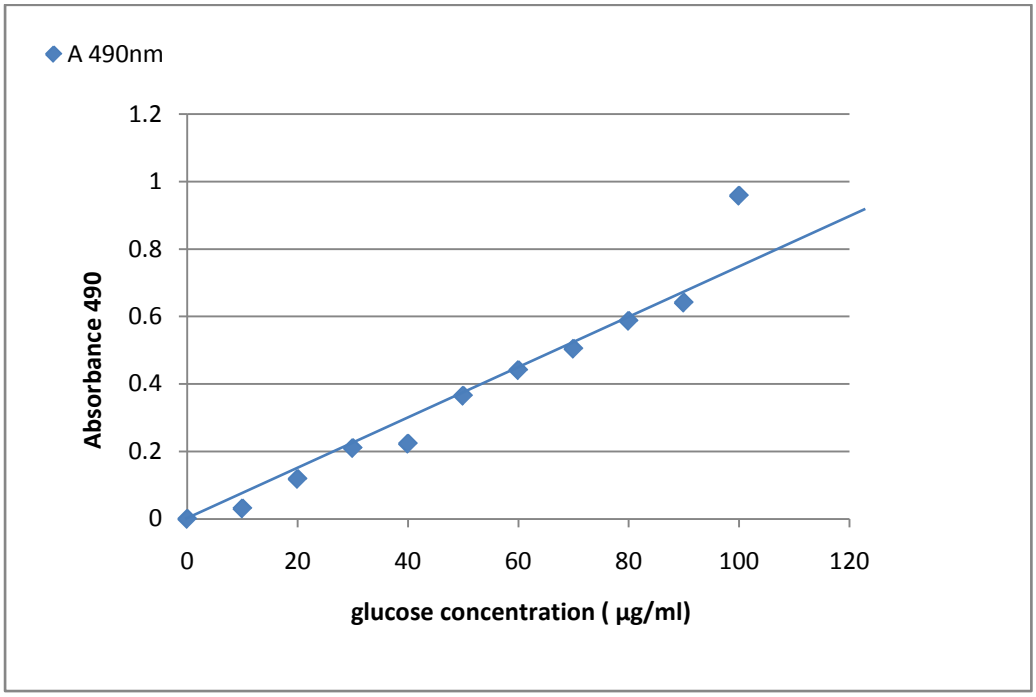


Fig. (2-2): Standard curve of glucose concentrationdetermind by Dubois *et al.*, (1956) method.

2.2.11ChorioallantoicMembrane (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 10-7 days-old fertilized eggs was incubated at 37.5 ◦C with 55% relative humidity. On the eighth day, a $1cm^2$ window was carefully

created on the broad side of the egg shell, which can candle the egg to assure existence of embryonic blood vessels. A volume of glucan (20µl containing 250, 500, 750, 1000 or 1500µg/egg) anddistilled water (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 eggshellwas pushed aside around the window, and the blood vessels were photographed. Anti-angiogenic effect of glucan on CAMs was quantified by counting the number of blood vessel branch points which were marked using artistic software on the photos.

2.2.12Phagocytosis Assay for the β-Glucan:

The assessment of the phagocytosis assay*in vitro* was performed according to the procedure described by Furth*et al*., (1985)as follows:

One ml of heparinized blood was mixed with 0.5 ml of the glucan different concentrations (2.1.7.6), mixed gently and incubated at 37°C for 30 min, then 1ml of the yeast suspension ($1x10^6$ cell/ ml) was added, mixed gently and re-incubated at37°C for 30 min.

Blood smears on glass slideswere prepared, left to dry, fixed with methanol by dropping 3-5 drops to cover the smear and left to dry. fixed cells were immersed in Sorenson's buffer for 5 min and then left to dry. The slides were stained by adding a mixture of freshly prepared Giemsa stain and Sorenson's buffer (1:4 v/v) for 5-10 min.The slideswere rinsed with Sorenson's buffer and let to dry. Finally slides were examined under light microscope (100X). The phagocytic and non- phagocytic cell were counted. Control samples were PBS with blood.

$$\textbf{Phagocytosis \%}=\frac{\textbf{Phagocytic cells}}{\textbf{Phagocytic cells}+\textit{non Phagocytic cells}}\ \textbf{x100}$$

2.2.13 Quantitative Detection of Serum Level of Mouse Vascular Endothelial Growth Factor(VEGF)

Serum levels of VEGF were quantitatively determined in mouse serumand control subjects by means of quantitative sandwich enzyme linkedimmunosorbent assay ELISA test using ready kit.

2.2.13.1 Experimental Animals

Groups of Whit mice (8-10week old, 20-25g in weight) were used throughout this study and were supplied from Ministry of Health – Drug Monitoring Center. These animals were housed in plastic cages and maintained in hygienic conditions at temperature around 25°C and fed with suitable quantity of water and complete diet.

This experiment was used to evaluate the effect of glucan on the level of mouse vascular endothelial growth factor. Seven groups of mice were used in this experiment and treated as follows:

Group I: Control (3 mice): treated with 0.1ml of phosphate buffer saline.
Group II: glucan treatment (3 mice), treated with 0.1ml of glucan (10mg/Kg).
Group III: glucan treatment (3 mice), treated with 0.1ml of glucan (20mg/Kg).
Group IV: glucan treatment (3 mice), treated with 0.1ml of glucan (30mg/Kg).

Group V: glucan treatment (3 mice), treated with 0.1ml of glucan(40mg/Kg).

Group VI:glucan treatment (3 mice), treated with 0.1ml of glucan (60mg/kg).

The phosphate buffer saline and glucan were administratedintraperitoneally for ten successive days, and the mice were sacrificed by the end of the tenth day. Blood samples were taken and allowed to clot for 2hrs at room temperature before centrifuged at 1,500 rpm for 20 min. Serums were aspirated and kept at -20°C until use.

For determination of mouse VEGF, the mouse serums samples were diluted (5-fold) into calibrator diluent RD5T before use as recommended by the manufacturer instruction.

2.2.13.2 Kit Components

- Mouse VEGFmicrotiter Plate, 2x96 wells pre-coated with polyclonal antibodies for mouse VEGF.
- Recombinant mouse VEGF standard 2.5 ng/vial (Lyophilized powder).
- Recombinant mouse VEGF control (Powder).
- Mouse VEGF conjugate 23ml/vial of a polyclonal antibody againstmouse VEGF conjugated to horseradishperoxidase with preservatives.
- Assay diluent RD1N 12 ml/vial of a buffered protein solutionwith preservative.
- Calibrator diluent RD5T 21 ml/vial of a buffered protein solutionwith preservative.
- Wash buffer concentrate (25X), 21 ml/vial of a buffered protein solutionwith preservative.

- Color reagent A, 12 ml/vial of stabilized hydrogen peroxide.
- Color reagent B, 12 ml/vial of stabilized chromogen(tetramethylbenzidine).
- Stop solution, 23 ml/vial of diluted hydrochloric acid.

2.2.13.3 Reagents Preparation

Before carrying out the assay procedure, the kit was left at roomtemperature (18-25ºC) for 30 minutes to equilibrate, as recommended by the manufacturer.

1. Mouse VEGF Control: the control was reconstituted with 1ml deionized or distilled water. It was used undiluted.
2. Wash Buffer:it was preparedby taking 20ml ofwash buffer andcompleting the volume up to 500mlusing deionized or distilled water.
3. Substrate Solution: it was prepared by mixing color reagents A and Btogether in equal volumes 15 minutes before use.
4. Mouse VEGF Standard: It was prepared by reconstituting the mouse VEGF standard vial with 5ml of calibratordiluent RD5T to prepare a stock solution of 500pg/ml. The stock solution was allowed to sit for a minimum of 5 minutes with gentle mixing priormaking dilutions for standard curve.

2.2.13.4 Assay Procedure

1) A serial 2-fold dilutions series were prepared from mouse VEGF standard stocksolution (0, 7.8, 15.6, 31.2, 62.5, 125, 250, 500pg/ml) using calibrator diluent.

2) An aliquot of 50µl of assay diluent RD1N was added to each well in the antibody pre-coated Micotiter plate.

3) An aliquot of 50µl from each mouse VEGF standard dilutions, controls and pre-diluted serum samples were added to the appropriate wells. The plate was gently mixed by tapping the plate frame for 1 min.The plate was covered with the adhesive strip and incubated for 2 hours at room temperature.

4) The wells were aspirated and washed with five cycles of washing using the 400µl washing buffer (1X) for each well.

5) Aliquot of 100µl of mouse VEGF conjugate was added to each well. The plate was covered and incubated for 2 hours at room temperature.

6) The washing step was repeated as in point 4.

7) Substrate solution (100µl) was added to each well. The plate was covered and incubated at room temperature for 30min.

8) Finally 100µl of stop solution was added to each well. The plate was mixed well and the optical density was read at 450nm using a microtiterplate reader.

2.2.13.5 Calculation:

A standard curve was depended (fig.2-3) to determine the amount of mouse VEGF in an unknownsample. The standard curve was generated by plotting the optical density obtained for each of the standard concentration on the vertical (Y) axis versus the corresponding mouse VEGF concentration (pg/ml) on the horizontal (X) axis.

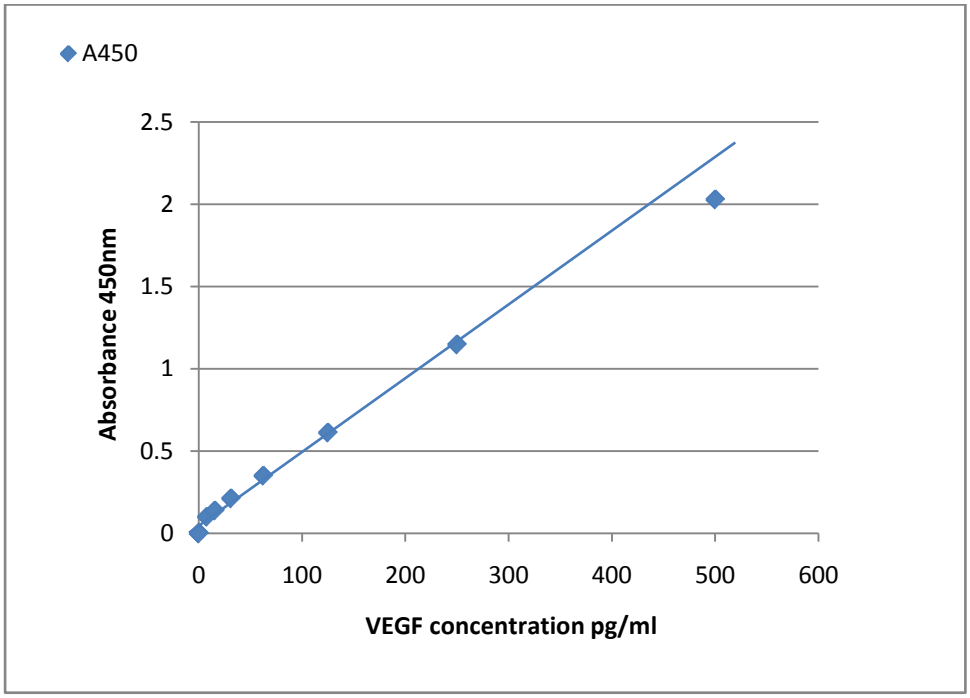


Fig. (2-3): Standard curve of mouse VEGF concentration

2.2.13.6 Statistical Analysis

A one way analysis of variance ANOVA (Duncan) was performed to testwhether group variance was significant or not. Data were expressed as mean ± standard error and statistical significances were carried out according to program statistical package for social sciences IBM SPSS, version 20.

Chapter three
Results and discussion

3. RESULTS AND DISCUSSION

3.1 *Saccharomyces cerevisiae*Isolates

*S.cerevisiae*has been isolated and purified from imported dry yeast,Safe-Instant(French origin) from local supermarkets.It was confirmed that the obtainedyeastwasbelonged to the species*S.cerevisiae* depending onmicroscopic, morphologicalcharacterizationand biochemical tests (table 3-1).

3.1.1 Microscopic and Morphological Characters

The yeast vegetative cellswerecharacterized by being oval to spherical in shape, clustered like beehives, contained large nucleus, large vacuole, buds found in more than one location and the absence of the true fungal hyphae**.**

Morphological characteristics of the isolate were observed, included the colonies shape, color and texture on the surface of YEPD agar. The colonies were circular in shape, white to creamy in color with smooth edge, convert, bowl textures, medium size; their diameter was about 1-2 mm.

3.1.2Biochemical Tests

Table(3.1) shows the results of the biochemical tests of the yeast isolate, thesetests have shown the ability of the isolate to ferment and assimilatemonosaccharaides and disaccharides which are: glucose, fructose, sucrose, maltose, lactose and raffinose.

The isolate wasunable to utilize lactose as carbon source, while fermenting and assimilating the other saccharides. In addition, the isolate also was unable to hydrolyzeurea and produce ammonia.The characters

are coinciding with *Saccharomyces cerevisiae* (Lodder, 1974; Barnett *et al*., 1990).

Table (3.1):The results of biochemical tests for *S. cerevisae*

Biochemical test		**Yeast isolate**
Carbone Source Fermentation	**Glucose**	**+**
	Fructose	**+**
	Sucrose	**+**
	Lactose	**-**
	Maltose	**+**
	Raffinose	**+**
Carbone Source Assimilation	**Glucose**	**+**
	Fructose	**+**
	Sucrose	**+**
	Raffinose	**+**
	Lactose	**-**
	Maltose	**+**
Hydrolysis of urea		**-**

3.2 Extraction of Glucan from *Saccharomyces cerevisiae*

In this study glucan was extracted using a method described by Byron (1993), which depended on basic-acidic extraction steps.This method is characterized by its ability to extract glucan from baker's yeast with significantly higher amount, limited use of organic solventsand time saving comparing with other methodsfor the extraction of glucan like saki1 and saki2 (Al-Rubaee, 2008).

By this method the dry weight of obtainedglucan was 8.8g/ 100g of the dry yeast.AL-Rubaee, (2008), reported the ability to obtain glucan 6.25g/100g dry weight baker's yeast by following the method described by Byron (1993) method.Glucan can be extracted from backer's yeast with varying degrees of purity depending on the method used; the chemicaland physical properties and biological activity may vary according to the method of extraction (Anthony *et al*., 2005).

Zechner-Krpan*et al*. (2010) showed that the yield of beta glucan from yeasts using alkaline – acid extraction was up to 12%. Many procedures used for the yeast's glucan extraction were based on alkaline – acid applications with differences in application time, the type and the concentrations of the chemicals.The advantage of using alkaline-acid extraction method was the treatment with base, acid and organic solvent which leads to dissolve or remove most the proteins, mannan, nucleic acids and other components.

The 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).

3.3 Analysis of GlucanStructure

3.3.1 Determination of Carbohydrate and Protein Content of Glucan

Chemical composition of the glucan extracted from*Saccharomyces cerevisae* were performed by estimating the carbohydrate and protein contents.

Results indicated that the percentage of carbohydrates and proteins of the extracted glucan were 44% and0.45%, respectively. The components of

both glucose and protein gave an important indication about glucan purity;therefore the high amount of sugarswithlow content of proteins indicating that the method of choice was good in yielding glucan with very small amount of impurities. The glucan purity is an important character in determination of its application since glucanmay be used in pharmaceutical, cosmetics, food and other felids(Vetvickaand Vetvickova, 2007).

Lee *et al*. (2001) reported the ability to yield 32% glucan and 0.8% proteins using alkaline – acid extraction method.

3.3.2 Analysis of Glucan by FT-IR

Glucan was analyzed using FT-IR spectroscopy to detect the functional group in its chemical structure of glucan, and compared these groups with standard ones.FT-IR technology is used for the organic molecule diagnosis by detecting the active groups and bounds found in the molecule(Ibrahim *et al*., 2006).

Result in Fig. (3.1a) showed that infrared spectrum at the absorbance 1041.5 cm^{-1}means the presence of C-O-C bonds which isa characteristic feature for β-glucan structure stretchingwith thestandard 1051cm^{-1} (Fig.3.1b) (Hozova*et al*., 2007). The absorbance at (1384.8 cm^{-1}) refers to the presence of C-H aliphatic bending;the standard absorbance was at 1375cm^{-1} (Karreman *et al*., 2006).

On the other hand,free hydroxyl groups and carboxyl groups were absorbed at regions 2862.2 cm^{-1} and 2923cm^{-1}that found in the carbohydrate (Ibrahim *et al*., 2006).

Result indicated that the FT-IR spectra of the extracted glucan had appearance typical to that of the standard β-glucan with high degree

ofpurity and absence of the protein contents that absorbed at 1635, 1542, 1650 cm^{1} (McCann *et al*., 1992).

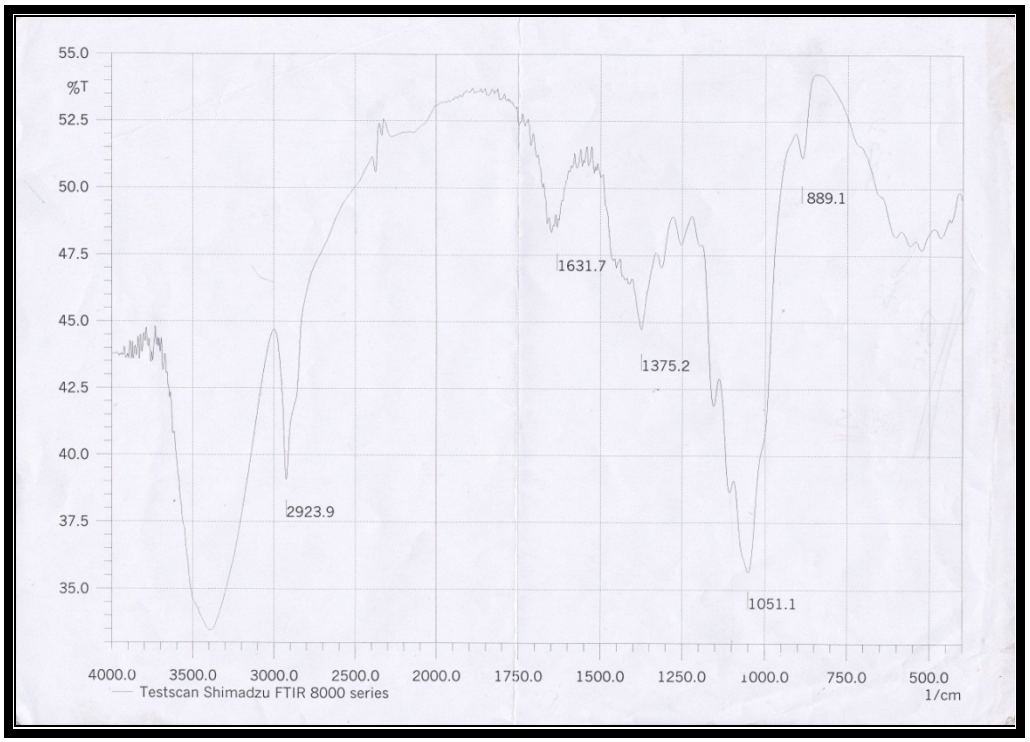


Fig. (3.1a): The FT-IR for *S. cerevisiae* glucan

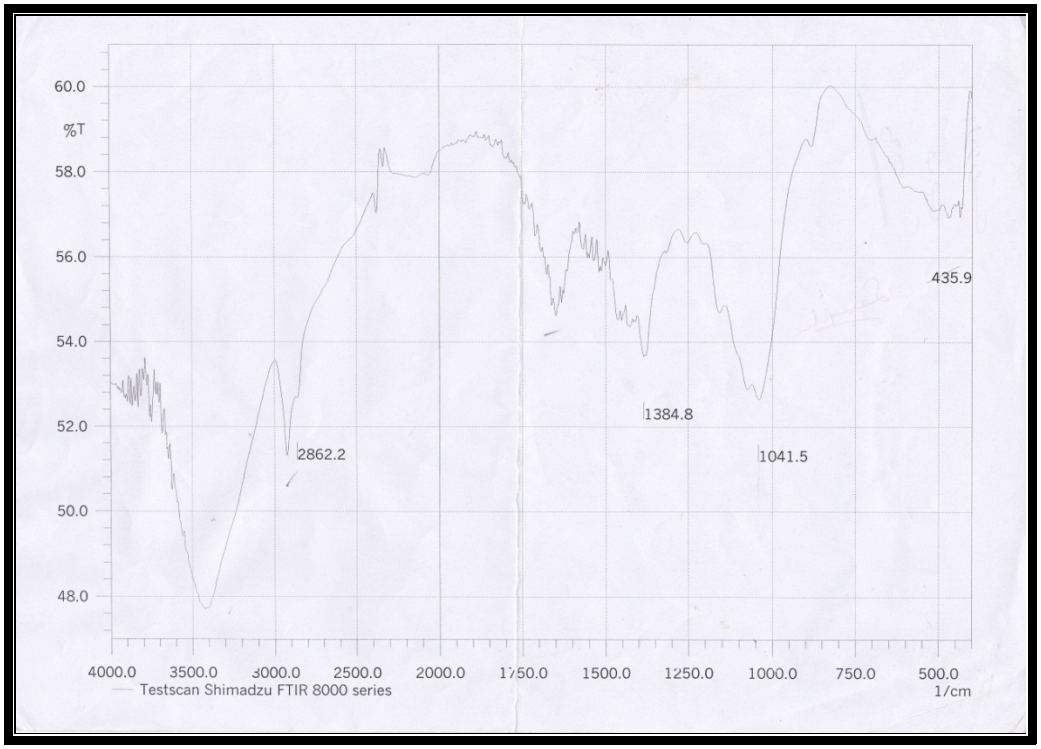


Fig. (3.1b): The FT-IR for *S. cerevisiae* standard glucan

3.3.3 Molecular weight of glucan

The molecular weight of the extracted glucan was determined by gel filtration chromatography usingSephacryl S-300 column.

The results in Figure3.2, showed the appearance of single peak near the void volume of the columnthis confirmed that no de-polymerization was occurredduring glucan extraction and that glucan had relatively high molecular weightof approximately 300kDa (Fig. 3.3).

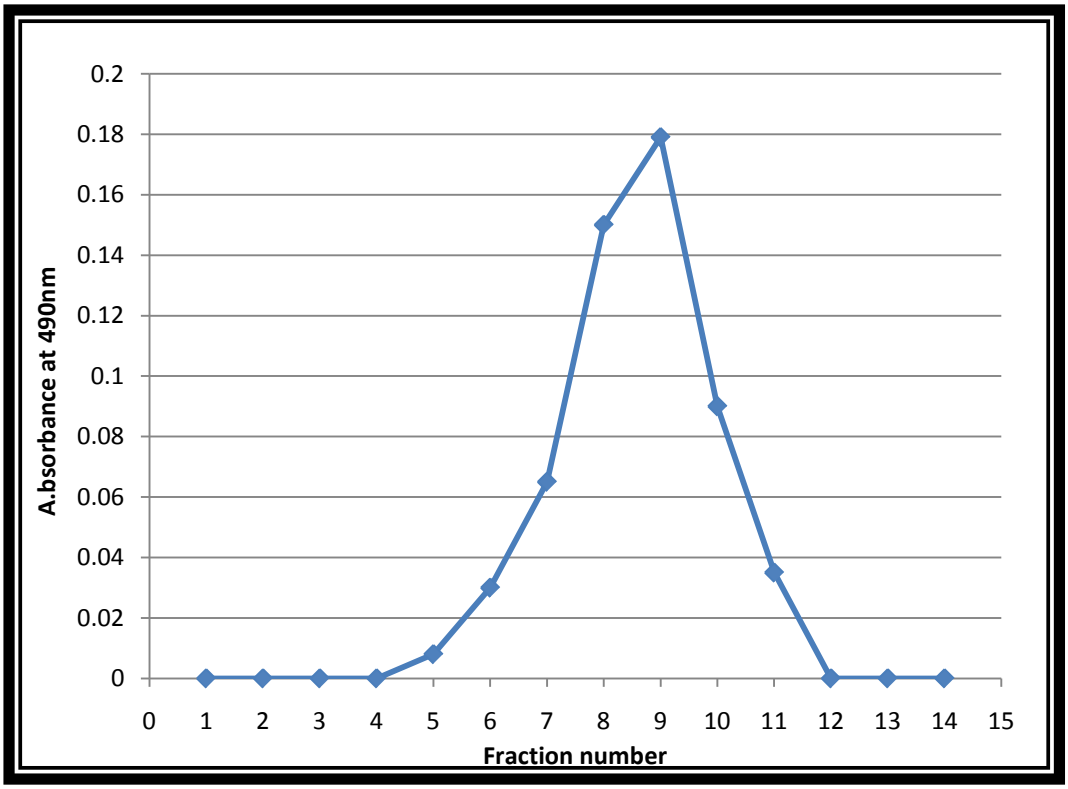


Fig. (3.2): Gel filtration of glucan bySephacryl S-300 column (2×32cm) eluted with BPS. Five milliner's fractions were collated at flow rate of 1ml/min assayed by Dubbios assay

The molecular weight of glucan extracted from yeast cells may vary according to its polymerization and this is highly depending on the source of glucan and extraction method (Manners*et al*., 1973). That is may explain why Manners *et al*. (1973)reported lower molecular weight of about 240 kDa for the glucan extracted from*S.cerevisiae*.The molecular weight is an important property to determine the efficient degree of β-glucan as

immunomodulator, whenever the molecular weight is more than 100 kDa it will be more efficient for activating of MNC and increasing the secretion of IL-12 from these cells that inhibit the angiogenesis (Yang*et al*., 2009). As a result, the molecular weight of the extracted glucan was more than 100kDa and thus it was candidate to be used in further immunological studies.

Different molecular weights for glucan were obtained from different medical fungi that can be used as antitumor and antiangiogenic compounds like glucan extracted from *Antrodia xanthan* which had molecular weight of glucan 304kDa and glucan extracted from *Rigidoporusulmarius*which was estimated to be325kDa (Chen *et al*.,2005).The molecular weight (300kDa) glucan from*S.cerevisae* can be considered to within the range of the effective molecular weights (300 – 325kDa) that have efficient effect on the blood vessel angiogenesis (Chen *et al*.,2005)

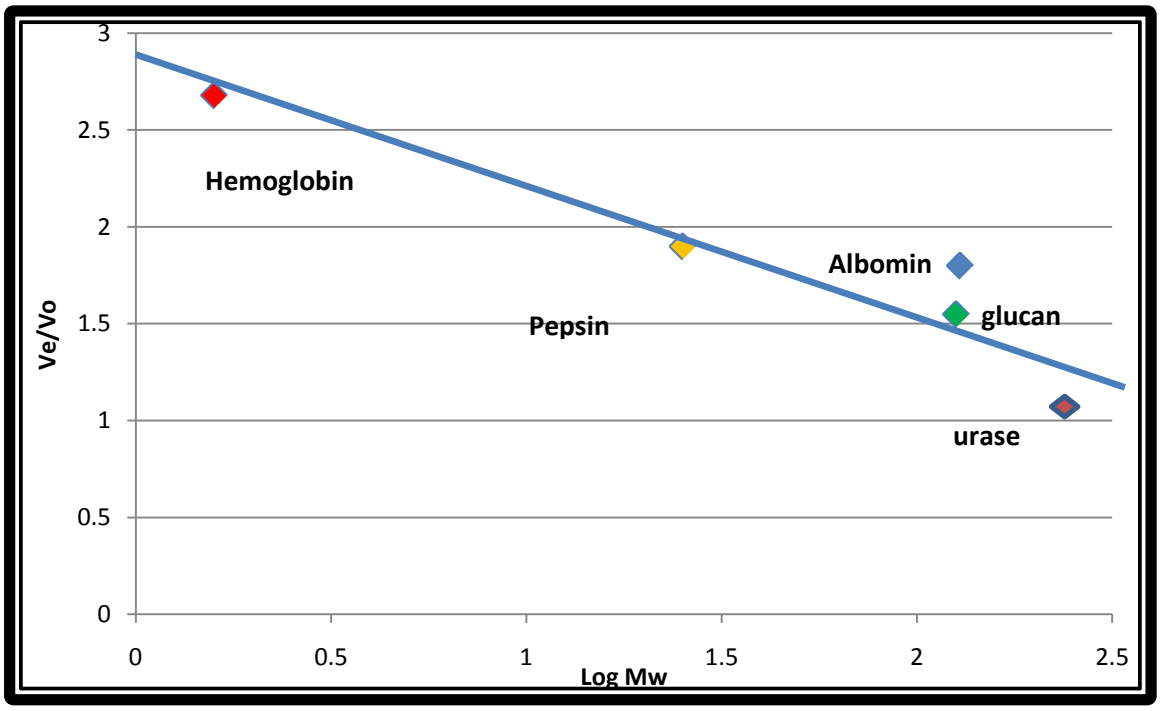


Fig (3.3): Standard curve for molecular weight determination of glucan by Sephacryl S-300 (V0: void volume, Ve: elution volume)

3.3.4 High performance Liquid Chromatographic Analysis of Glucan

In this study, this technique was used to determine the quality and purityof*S.cerevisiae*beta glucan, in addition to confirm the structural similarity with the standard beta glucan.The HPLC analysis revealed one major peak 3.78 of a liquid sample glucan(Fig. 3.4a), which indicating the purity of the extracted glucan. Such peak showed the same retention

time of the glucan standard (Fig. 3.4b) and (Fig. 3.4c)indicating the efficient method of the extraction.

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).

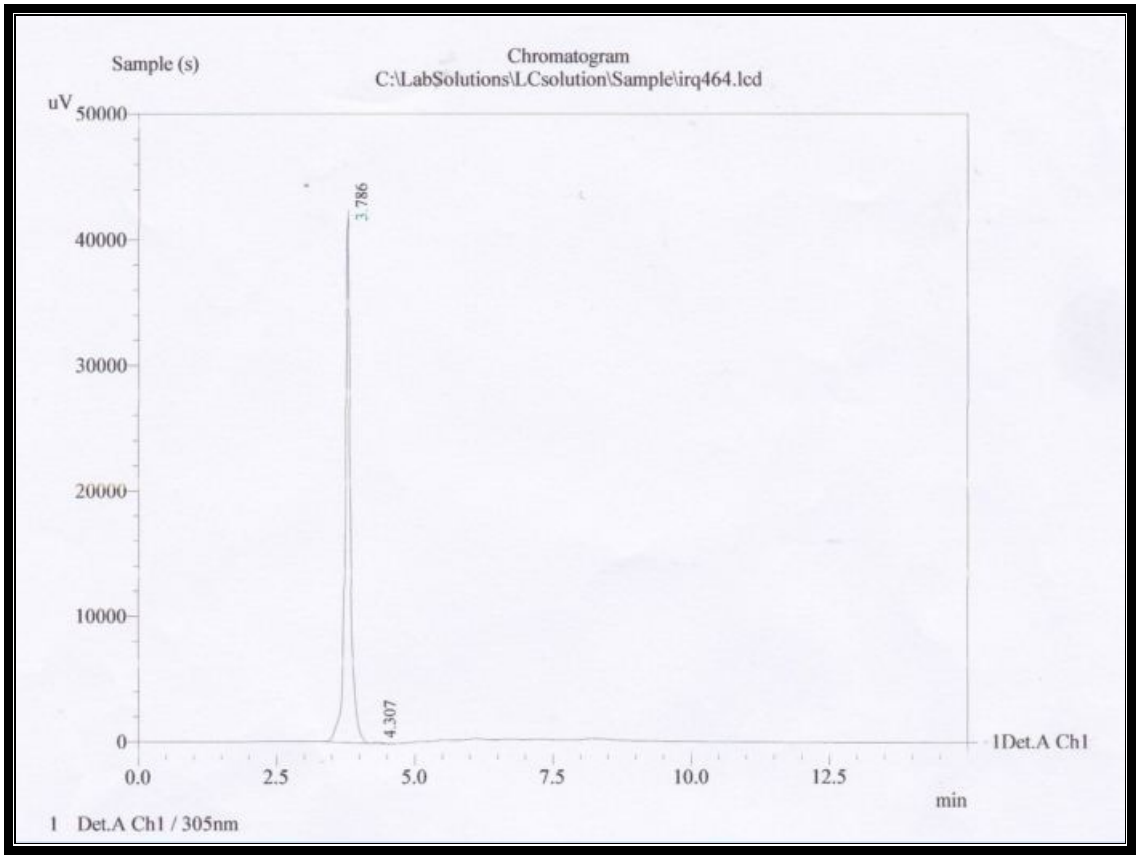
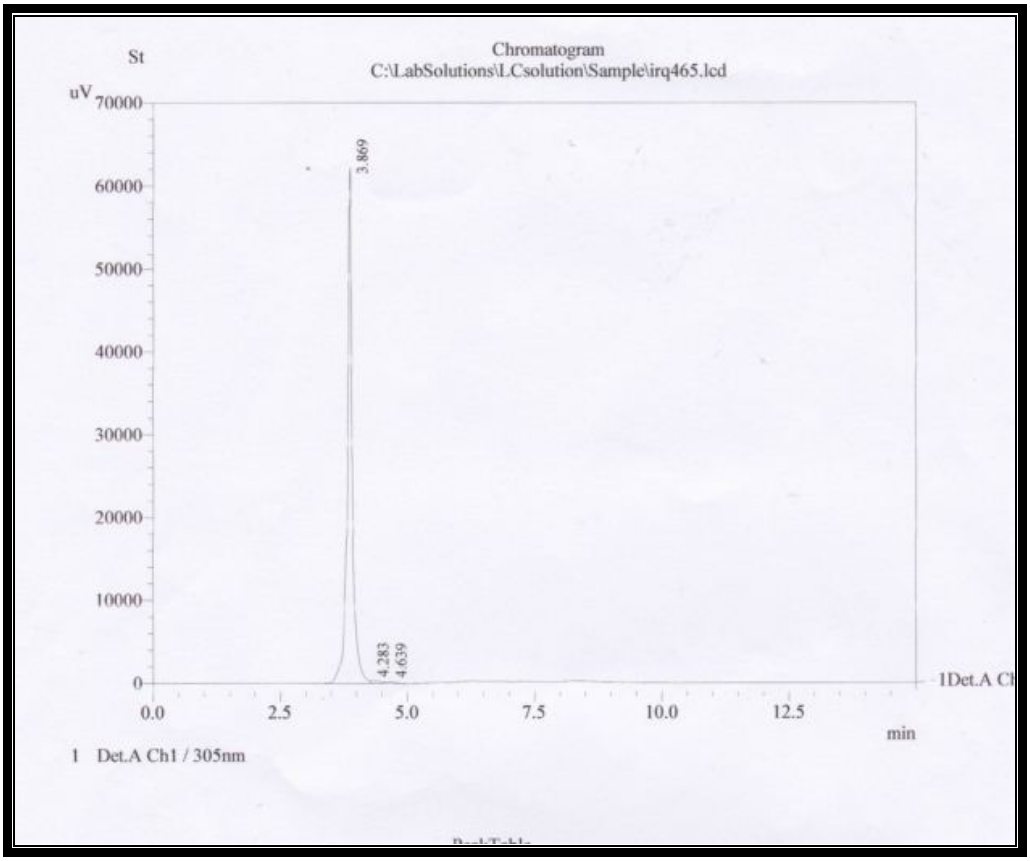
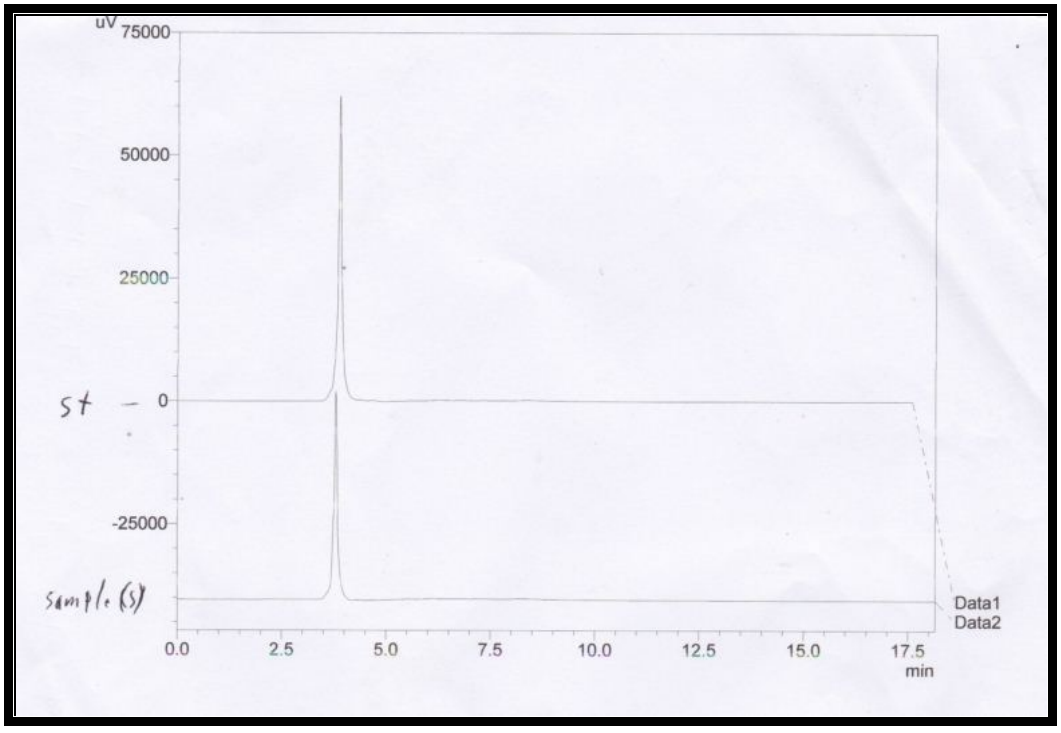


Fig. (3.4a): HPLC analysis for *S. cerevisiae*glucan sample



(Fig.3.4b) HPLC analysis for *S.cerevisiae*standard gluca



(Fig.3.4c) HPLCanalysis for comparison of glucan sample and standard

3.4 Anti-angiogenicEffect of *S.cerevisiae*β-Glucan

The anti-angiogenic activity of the*S.cerevisiae* β-glucan was detected by CAM assay. Table3- 2 revealedthe result of this assay.

Table(3.2):Effect of β-glucan on the neovascularizationof the chick embryo

Concentration of glucan µg/egg	Numbers of blood vessel branches Mean±SE
Negative Control(0)	60.3±1.45 a
250	58±0.57 a
500	53±1.15 b
750	50±0.57 c
1000	43±0.57 d
1500	35±0.57 e

Letters' b, c,d ,e refers to significant result (Significant Value is $p \leq 0.05$)

According to the results, the concentration 250µg/egg of β-glucan had a slight non-significant inhibition effecton neovascularization as compared with the negative control and the number of the blood branches was 58±1.45 (Figure 3.5b).

On the other hand, the concentrations (500, 750 and 1000µg/egg) revealed a significant ($P \leq 0.05$) inhibition of the

neovascularizationprocess with a decrease in blood vessel branches 53, 50 and 43, respectively(Fig. 3.5 c, d, e).

It was noticed that the increase in the concentration of β-glucan, will inversely decreased the chick CAM angiogenesis and by reaching the concentration of1500µg/egg, 50% of CAM capillarizations were significantly($P \leq 0.05$) inhibited (Fig. 3.5 f).

Results indicated thatβ-glucan extracted from *S. cerevisiae* affects the generation of the new blood vessels and hence reduces the number of blood vessels branches.The decrease of the neovascularizationmay be attributed to several lines of evidence that might support the role of polysaccharides (β-glucan) in anti-angiogenisisprocess.

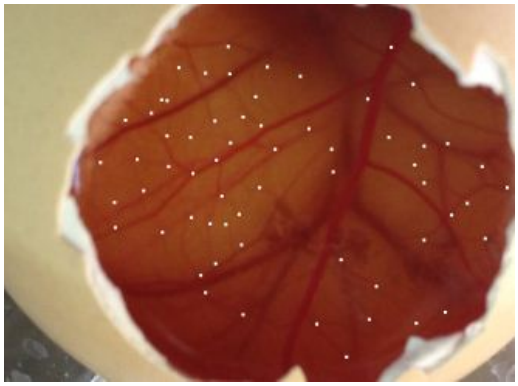
β-glucan is most effective in promoting the secretion of IL-12 that has been shown to directly inhibit angiogenesis of endothelial cells (Del Vecchio*et al*., 2007), β-glucan injection intraperitoneally to the mice caused a decrease in VEGF level and leads to the activation of the MNC to secret IL-12(Yang*et al.*, 2009).

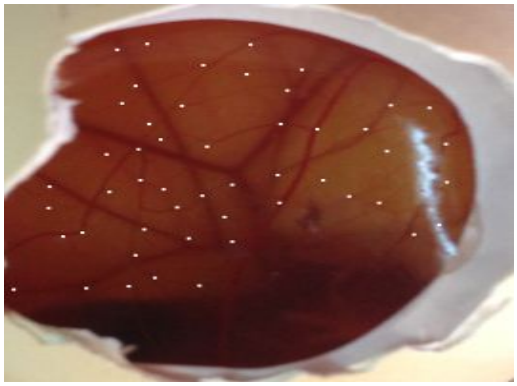
The VEGF is afactor that regulates the formation of blood vessels, injection the mice with concentration 500, 750, 1000; 1500µg/egg of glucan decreased VEGF and inhibited the neovascularization (Del Vecchio*et al*., 2007).

There is a relationship between the molecular weight and anti-angiogenic effect of the polysaccharides, in whichwith high molecular weights (2693 - 2876 kDa) and mid molecular weights (300 – 325kDa) have anti-angiogenic effect on the blood vessels, the polysaccharidewith middle and high molecular weight inhibit the endothelial tube formation and hence inhibit the process of angiogenesis (Chen *et al*., 2005).

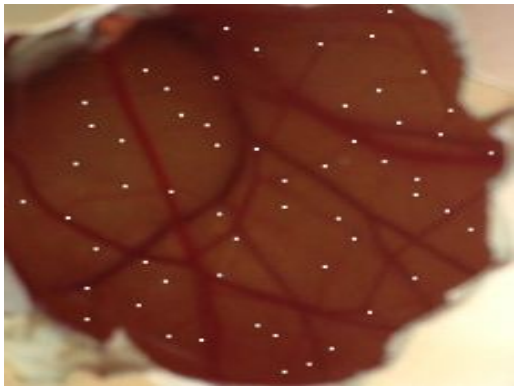
Many studies utilized the glucan of fungi as anti-angiogenicfactor;Chen*et al.*(2005) used different molecular weights ofdifferent medical fungi glucan as anti-angiogenic factor.

Seung*et al.* (2006) extracted the beta glucan from *S.cerevisae*withanti-angiogenic agent.Yang*et al.*(2009) showed that the polysaccharide from the *Antrodiacinnamomea*confirmed its anti-angiogeniceffect.

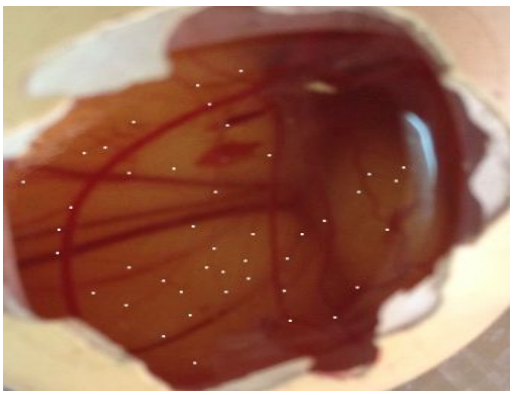




(A) (B)



(C) (D)



(E) **(F)**

Fig. (3.5): The effect of*S.cerevisiae* β-glucan concentrations on the neovascularization of the chick embryo blood vessels

A- Control (0) **B-250µg/egg** **C-500µg/egg**

D-750µg/egg **E-100µg/egg** **F-1500 µg/egg**

(The dotes in the figures indicated the number of the blood vessels branches)

3.5The *in vitro*Effect of β-Glucan on Phagocytosis

The effect of blood phagocytic cellsagainst yeasts cells was examinedwith the presence of the extracted β-glucan Table (3-3).

Table (3.3):Effect of β-glucan on the phagocytosis toward yeast cells

Concentration of glucan µg/ml	Phagocytosis% Mean±SE
Control(0)	**50.8±3.4 a**
250	**51.2±1.7 a**
500	**60.7±1.2 b**
750	**61.9±0.9 b**
1000	**62.5±1.7 b**
1500	**70.7±0.6 c**

Letters' [b, c]refers to significant result (Significant Value is*p*≤0.05)

Result in the Table 3-3indicatesthat the phagocytosis coefficient values increased significantly($p \leq 0.05$) with increasing β-glucan concentrations.The highest rate of the phagocytosis coefficient reached up to70% at the concentration 1500µg/ml, while there is no effect at the concentration 250µg/ml.However,with concentrations 500, 750, 1000 µg/ml the effect was no significant ($p \geq 0.05$) higher than the control and 250 µg/ml but no differences in effect recorded between these concentrations.

It's clear that β-glucan has the ability to activate the phagocytic cells and increase their ability to engulf the dead yeast cells when the blood phagocytic cells interact with β-glucan.Thephagocytosis is one of

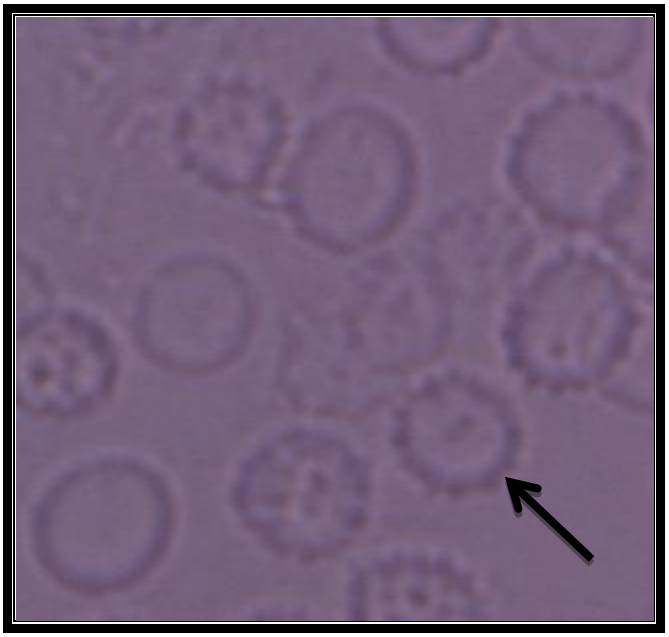
the mechanisms of the bodydefense against the pathogen; monocyte, macrophage and polymorphic nucleus cells are the main cells in this respect and they represent as the first line of the body defense, they found in most of the body tissues (Vetvicka and Vetvickova, 2007).

β-glucan is one of the immune modulators especially that extracted from microorganisms.The immune modulators have the ability to activate the immune cells in order topass in the states of activation leads to production of some compounds that play important roles in immune organization like(IL-12 and TNFα) in addition to inflammatory mediators which increase the ability of the immune cells to defense against pathogens (Vetvicka and Vetvickova, 2008).

β-glucans is recognized as pathogen-associated molecular patterns (PAMPs) by several mammalian immune cell receptors, such as dectin-1, toll-like receptors, complement receptor 3(CR3).These receptors allow the β-glucan to interact with immune cells, such as macrophage, neutrophils and lymphocyte;such interactions will activate several intracellular pathways responsible for the immune pharmacological properties (Medeiros*et al*., 2012).

Macrophage is one of the immune cells that recognize the β-glucan and play critical role in all phases of the host defenses that are both innate and adaptive immune responses, the secretions of (IL-12and TNFα) and inflammatory mediators are affected by the macrophage cellsactivation, therefore the activation of these cells byβ-glucan increases host immune defense (Dalia *et al*., 2007).

Al-Rubaee(2008) indicated that increasingin phagocytosis activity for thepolymorphic nuclear cells(PMNc) up to 74%when yeast beta glucan was used as modulator.AL-Aubydi *et al*., (2011), depended on βglucan as immunomodulator and indicated that the β-glucan have a positive effects in animals immune system.



Fig(3.6)Blood film of phagocytosis test sample showing the the phagocytic cells engulfed yeast cells (100X)

3.6 Effect of β-glucan on theVasclar Endothelial Growth Factor(VEGF)

This assay was conducted to determine the effect of β-glucan on the secretion of the VEGF using an ELISA kit (R&D mouse VEGF ELISA kit)

Table (3-4) effect of β-glucan on the VEGF

Concentration of glucan Mg/Kg	Concentration of VEGFpg/ml Mean±SE
Control(0)	195±3.3 a
10	200±3.3 a
20	130±6.3 b
30	110±13.3 b
40	85± 3.3 c
60	85± 3.3 c

Letters[a, b, c,d] refers to significant result (Significant Value is$p \leq 0.05$)

The coefficient value of the VEGF concentration decreased with increasing of β-glucan concentration table(3-4)shows the result.There is no significant ($p \geq 0.05$) effect of the β-glucan at the concentration 10mg/Kgin comparison with control;however the effect of β-glucan started from 20 and 30mg/Kg, with a moderate effect on the production of mouse VEGF, while40 and 60mg/Kg, which revealed the maximum significant($P \leq 0.05$) effect on the secretion of the VEGF that caused a decreasing in the concentration of the VEGF to 85pg/ml.

Under *in vitro* conditions, VEGF stimulates growth of endothelial cells originated from arteries, veins and lymphatic vessels by direct action on them.VEGF is a powerful inducer of angiogenesis in many experiments, itprevents apoptosis of the endothelial cells, and it has been shown that VEGF suppressthe expression of the apoptotic proteins Bcl2 and A1 in the endothelial cells (Karamysheva, 2008).

When the level of the VEGF deceasesit will cause adecreasesin the angiogenesis process by promoting the apoptotic proteins to do their jobs and subsequently decreasing the endothelial cells and the neovascularization.

The injection of the β-glucan of *S.cerevisiae* into the mice caused decreasing the level of VEGF by promoting the serration of the IL-12 and directly inhibits the angiogenesis of the endothelial cells by inhibiting the formation of the endothelial tube formation (Yang*et al*., 2009).It was found that β-glucan extracted from the mycelia of the *Coiolusversicolor* canreduce tumor weight and vascular density via suppression of the vascular endothelial growth factor gene expression (Shyur and Allan, 2012).

There are many studies used the β-glucan for reducing the secretion of the VEGF, suchpolysaccharides extracted from medically important fungi showed a reductionin the VEGF level and inhibited the formation of blood vessels(Chen*et al*., 2005). Such effect also detected regarding polysaccharides extracted from *Antrodiacinnamomea*mycelliathat showed a decrease in VEGF level in human umbilical vascular endothelial cell (Yang *et al*., 2009).

Chapter four
Conclusions and recommendation

CHAPTER FOUR: CONCLUSIONS AND RECOMMENDATION

4.1 Conclusions

1. β-glucanwas extracted from local dried baker yeast *Sacchromysescerevisae*usingacid –basic hydrolysis method with dry weight 8.8g/100g, high purity (Carbohydrate and protein contents were 44% and 0.45% respectively)and molecular weight of 300kDa.
2. The HPLC and FT-IRanalyses confirmed that the extracted glucan was β-glucan as compared with the standard.
3. β-glucan showed the ability as anti-angiogenic effectorat the concentrations of500,750,1000,1500µg/egg, with apparent decrease of the neovascularization.
4. The phagocytic function of blood phagocytic cells increased the presence of β-glucan that reached to 70% at the concentration of1500µg/ml of the extractedβ-glucan.
5. The β-glucan injection in mice caused a decrease in the level of the mice serum VEGF at the concentration of 1500µg/ml.

4.2 Recommendations

1. Introducing and modulating new methods for the extraction the β-glucan involved extraction of β-(1, 6) that neglected in many types of extraction of glucan.
2. Chemical modifications or conjugate preparations of the glucan compound to be more effective for increasing the immune response.
3. Studying the cytotoxic effect of the extracted β-glucan on tumor cells *in vivo and in vitro*.
4. More thorough studies on the application ofβ-glucan as anti-angiogenic factorinvolving more *in vivo* studies including tumor model mice.
5. Studyingthe effect of the β-glucan on the other types of cytokines and factors that involve in immune response and may directly and indirectly affect the angiogenesis process.
6. Studying whether β-glucan exerts antioxidant effect.

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الخلاصة

حددت نسبةعامل النمو البطاني الوعائي (VEGF) للفئران المختبرية بعد حقنها تحت غشاء البريتون بتراكيز مختلفة من الكلوكان. لوحظ ان الكلوكان له القدرة في تقليل نسبةعامل النمو عند التراكيز(١٠,٢٠.٣٠,٤٠,٦٠ mg/Kg) بينما التركيز١٠ mg/Kg لم يظهر اي تأثير معنوي في تقليل نسبه عامل النمو في الفئران المحقونة.

الخلاصة

أجريت هذه الدراسه بهدف التحري عن تأثير β-glucan في منع تكوين الاوعية الدموية وتأثيراته المناعيه. جمعت نماذج الخميرة *Saccharomyces cerevisiae* منالاسواق المحلية في العراق. أكدت الفحوصات المجهرية والتشخيصات الزرعية والفحوصات الكيموحياتية ان العزلة تعود لخميرة*Saccharomyces cerevisiae* .

تم استخلاص الكلوكان من الخميرةحيث اعطت وزن جاف بلغ 8.8g/100g. قدرت نسبة السكريات والبروتينات للكلوكان المستخلص و قد اعطت نسبة تبلغ 44% و0.45% بالتتابع .

أسخدمت تقنية تحليل طيف الاشعة الاحمراء FT-IR لتحليل الكلوكان المستخلص من خميرة الخبز واعطت النتيجةتشابه ودرجة نقاوة عاليه للكلوكان مقارنة للكلوكان القياسي وخلو الكلوكان من السكريات الاخرى مثل المانان والكلايكوجين.من ناحية اخرى اكد تحليل HPLC ان المستخلص يعود الى β–glucan والذي يحوي وقت استباق (retention time) مشابهه للكلوكان القياسي.

كما تم تقدير كذلك الوزن الجزيئي للكلوكان المستخلصبواسطة استخدام الترشيح الهلامي gel filtrationمن نوع SephacrylS-300وكان مقداره300 كيلو دالتون.

أجريت طريقة (CAM Assay) لتحديد قابلية الكلوكان في تثبيط تكون الاوعية الدموية في الجسم الحي وذلك بأستخدام البيض المخصب بعمر 8 ايام. تم استخدام عدة تراكيز من الكلوكان (1500, 1000, 750, 500, 250 µg/egg) حيثأظهرت النتائج مقارنة بالسيطره السالبه ان التراكيز (1500, 1000, 750, 500 µg/egg)اثرت بصورة واضحة في تقليل الاوعية الدمويه فيما اظهر التركيز 250µg/egg عدم تأثيره في تثبيط تكوين الاوعية الدموية.

تم تحديد تأثير اضافة الكلوكان بتراكيز مختلفة على عملية البلعمة للخميرة من قبل الخلايا الالتهامية في الدم حيث اثبت النتائج ان اضافة الكلوكان بتراكيز (1500, 1000, 750, µg/ml 500) له تأثيو واضح في زيادة نسبة البلعمة وكان تركيز 1500µg/mlقد اعطى اعلى نسبة في عمليه البلعمة بلغت 70% الا ان التركيز 25µg/mlلم يظهر اي تأثير معنوي في زيادة عملية البلعمة

بِسْمِاللّٰهِالرَّحْمٰنِالرَّحِيمِ

قَالُوا سُبْحَانَكَ لا عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ

سورة البقرة الاية (٣٢)

صدقَاللّٰهُالعظيمُ

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

تأثير مستخلص بيتا كلوكان من خميرة الخبز

في تكوين الاوعيه الدمويه

Saccharomyces cerevisiae

رسالة

مقدمة الى مجلس كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة الماجستير في علوم في التقانة الاحيائية

من قبل

هبة خليل ابراهيم
بكلوريوس احياء مجهرية – قسم علوم الحياة – جامعة بغداد (٢٠٠٢)

بأشراف

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٢٠١٤ — **١٤٣٥**