5-1 Conclusions

1. There is an obvious antibacterial activity for the crude polysaccharide extract of *Pleurotus* oyster (local and commercial isolates) against gram-positive and gram-negative bacteria.
2. The crude polysaccharide extract of local isolate (*Pleurotus* oyster) exhibit a remarkable immunomodulating effect for the host immunity.
3. A good antitumor activity exhibited by the crude polysaccharide extract of the local isolate of *Pleurotus oyster*.
4. *Pleurotus oyster* (local isolate) polysaccharide extract has a good stimulating effect on growth of normal cell culture.

5-2 Recommendations

1. Performing further studies on the biological activity of *Pleurotus oyster* polysaccharide extract.
2. Performing studies on treatment of different cancer patients by *Pleurotus oyster* polysaccharide extract. Since polysaccharide has no side effect compared with chemotherapy, which decrease the body's general defense mechanism.
3. Increasing consumption of mushroom because it may avoid the infection with many diseases.
4. Further purification for the crude polysaccharide extract and detect which is portion has the biological activity.

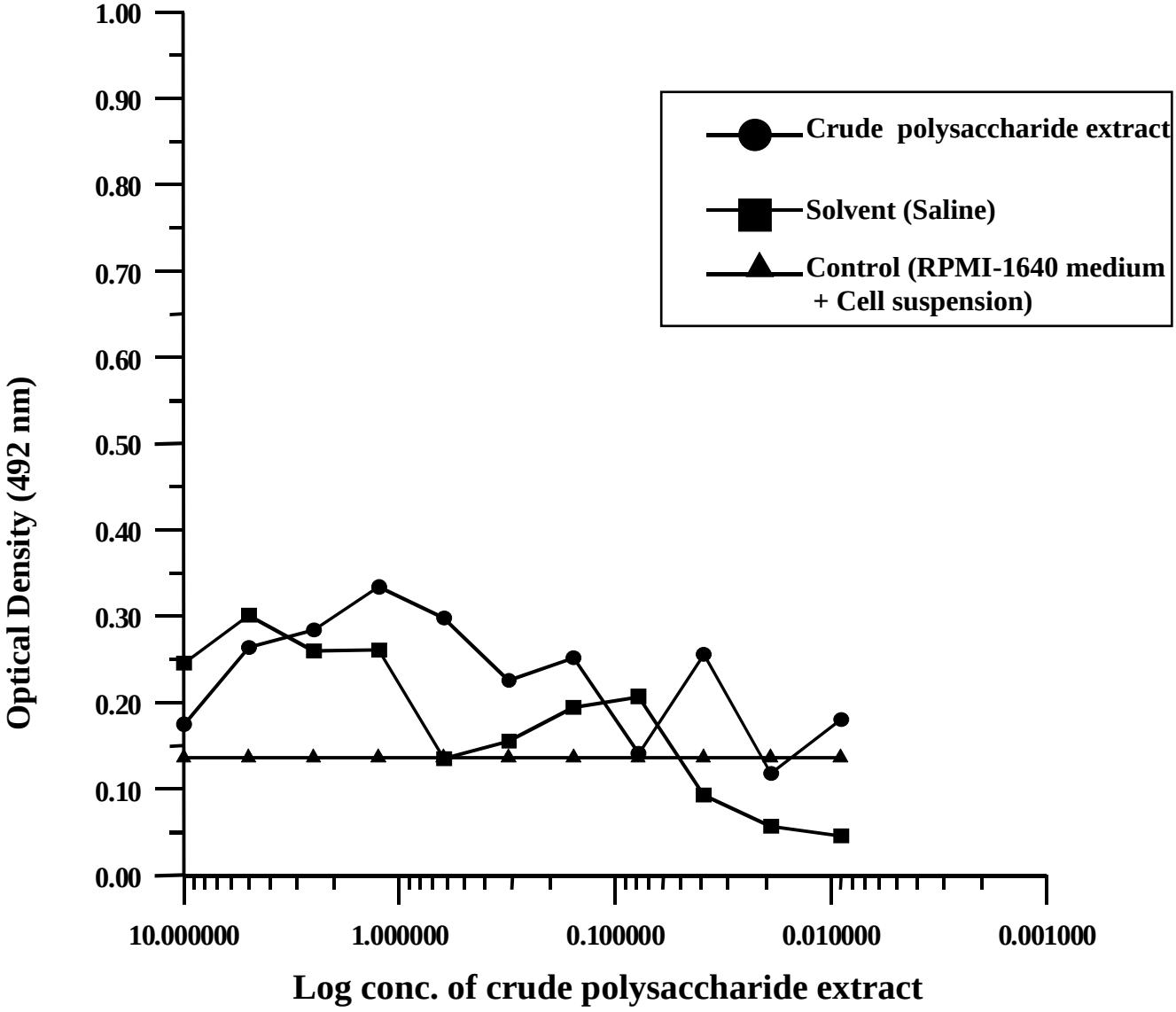

Figure (4-2) Cytotoxic effect of wild *Pleurotus oyster* crude polysaccharide on chick-embryo fibroblast cell

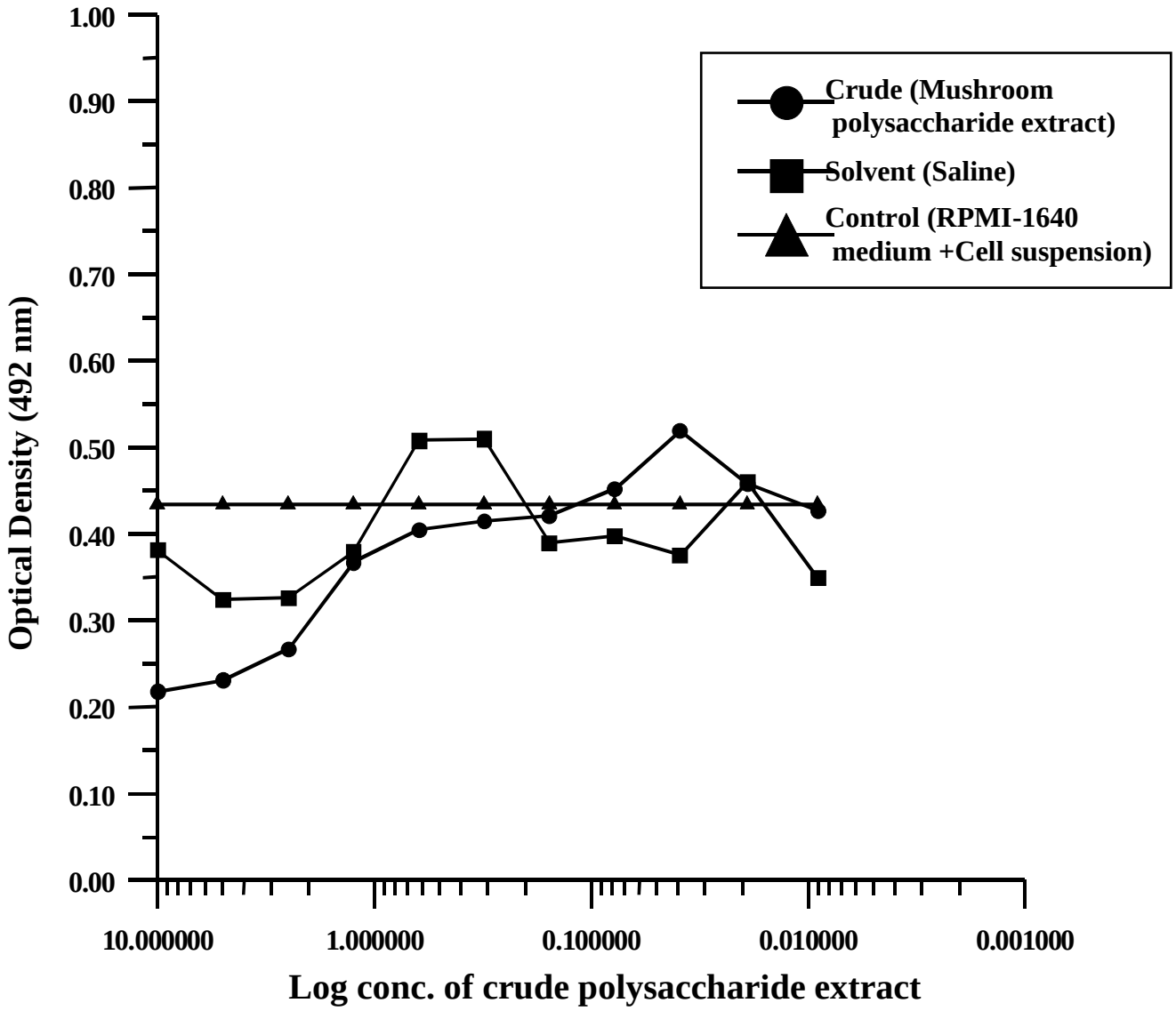

Figure (4-3) Cytotoxic effect of wild *Pleurotus oyster* crude polysaccharide on myloma cell-line.

Introduction

The use of botanical products in drug development involves the identification and extraction of active components.

The therapeutic use of plant extracts is among the oldest medicinal practices. It is a central feature of many forms of folk and traditional medicine, and it is used in the treatment of many disorders. Botanical products are also the source of much of the mainstream pharmaceuticals in the world [Bensky Gamble , 1993].

Mushroom therapeutically offers effective new tools for maximizing personal health in the human population without the toxic side effects common in many of the conventional treatment [Gordon *et. al.*, 1998].

Recent studies on mushroom extracts revealed their antimicrobial, anticholestrolic and tumoricidal effect of such extracts, and they revealed that the hot water extract exhibits potent anticancer activity [Ebina *et. al.*, 1998].

Chromatographic purification of mushroom extract showed that the main antimicrobial and tumoricidal activity in fractions containing alpha-1,4-glucan-beta-1,6-glucan complex. The crude mushroom extr-acts and its fractions showed an *in vitro* selective cytotoxic for tumor cell line, and having no effect on normal cell line [Fujimiya *et. al.*, 1999].

Since currently available anticancer agents have not compacted significantly on the survival of the majority of the patient with tumor, novel therapeutic stratigies are needed. One of these therapeutic

strategies is using polysaccharide extract of mushroom [Eckhardt, 1996].

In Japan, Russia, China and USA several different antitumor polysaccharide was developed from fruiting bodies and culture medium of various medical mushrooms like: *Lentinus edodes, Pleurotus oyster* and *Ganoderma lucidum* [Wasser *et. al.*, 1999]. Relatively few studies are available on the biological activity of mushroom polysaccharide extract [Britten *et. al*., 2000].

So this study aimed to extraction and purification of mushroom polysaccharide using local and commercial isolates of *Pleurotus oyster* mushroom, and then tests its biological activities.

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Literature Review

2.1 Mushroom as Plant Derived Drug

Natural products served humankind as the source of all drugs and higher plants provided most of these therapeutic agents. Today, natural products and their derivatives still represent over 50% of all drugs in clinical use. Higher plant-derived natural products representing 25% of them [Balandrin *et. al*., 1993]. Further evidence of the importance of natural products is provided by the fact that almost half of 25% best pharmaceuticals were either natural products or their derivatives [O'Neill *et. al*., 1993].

Mushroom extracts represented one kind of plant-derived natural drugs.

There are several types of fungi such as; black bread mold, sac fungi, club fungi and others. Mushroom is one kind of club fungi which has an economically importance [Sylvia, 1998]. There are many nutritional kinds of mushroom, for example: *Agaricus bisporus* (white bottom mushroom), *Pleurotus* species, *Lentinus edodes* (Japanese or shiitake mushroom), *Volvariella volvacca* (Chinese or straw mushroom) and *Auricularia* species [Al-Doori, 1996].

The use of mushroom in food and medicine dates back to 2000 years ago, and today more than 1.2 million metric tons are produced annually. There are about 10,000 known species of mushroom and about 700 of these have been determined to be edible [Wasser and

Wies, 1999]. The nutritional kind of mushroom characterized by its good flavor, taste, and contianance of all essential components of perfect, balanced food [Al-Doori, 1996]. The edible mushroom contains low rate of lipids so it is representing an important food for fatty people, heart disease and hypertensive patients [Khan and Kausar, 1981; Sohi, 1988; Flegg, 1992; Chelawat and Choudhary, 1994].

Pleurotus mushroom characterize by its simple and low cost of technical production and high protein content that exceeded a lot of edible fungi [Chang *et. al*., 1981; Rajarathanam and Bano, 1988] *Pleurotus, Reishi, Maitake, Cordycepes,* and *Coriolus* mushrooms are of particular interest. These types produce a variety of complex active compounds which have been shown many functions such as:

a) Increased survival of gastric cancer patients.
b) Antibacterial, antiviral, and antiparasitic action.
c) Immunomodulatory compounds.
d) Block the carcinogenic activity of N-nitro compounds.
e) Improved liver function in hepatitis patients.
f) Stimulate and regulate the cardiovascular and respiratory systems.
g) Protect against radiation.

The exact mechanism of action of medicinal mushroom is only now beginning to be elucidated. Much of the activity is likely due to the presence of complex polysaccharides such as β-glucans which have cell surface activity. The action of these polysaccharides on tissue communication via cytokines and other biochemical intermediates

[Internet I]. Immunoceuticals can be considered as substances having immunotherapeutic activity when taken orally. More than 50 mushroom species have yielded potential immunoceuticals that exhibit anticancer activity *in vitro* or *in vivo* in animal models. For many years, humanity continuously looking for larger life span and better quality of life, but cancer continues to be scourge of humanity and leading to early death; now a day another dimension of anticancer therapy is available [Internet IV].

Immunotherapy means the body immunodefences that can carry out their natural function of eliminating abnormal tissue from the body. The tools of immunotherapy are naturally-occurring substances which can be included in the general category of dietary supplements, and mushroom has been recognized for its medicinal and antitumor activity [Borchers *et. al*., 1999]. *Pleurotus* mushroom containing antitumor substances found inside its cap that obtained by extraction methods. This extract have been found that it have an inducer effect in the formation of interferon which is used in viral infection treatment [Rambelli and Menini, 1985; Quimio *et. al* ., 1990]. Because of the nutritional and therapeutic importance of *Pleurotus* mushroom it have been become the second nutritional fungi that have been produced in the world after *Agaricus bisporus* [Flegg, 1992; Flegg 1994].

2.2 Extractions and Purification of Crude Mushroom Polysaccharide Extract

The crude polysaccharide mushroom extract was isolated by several extraction methods such as hot water extraction, 5% sodium hydroxide, ammonium oxalate, and using acetic acid .

The most useful extraction method is hot water extraction because of its easy to apply, their chemicals are very widely used, and finally the crude polysaccharide extracted by this method having a good and strongly biological activities [Internet II].

The aim of purification was to separate a certain compound from a pool of compounds in crude extract.

The purified compound is a result of series of separating methods which were either based on the surface charge distribution such as precipitation by high concentration salts like ammonium sulphate, or by using solvent such as ethanol or acetone which both of them were based on the reduction of all dielectric constant of the solvent, so the aggregation were likely to be electrostatic and dipolar vanderwaals forces [Whitaker, 1972], or based on the charge density such as ion-exchange chromatography and electrophoresis or based on the molecular weight like ultra filtration, dialysis, and gel filtration [Suelter, 1985; Scopes, 1987; Stellwagen, 1990].

The purpose of purify the crude polysaccharide extract is to obtain the most active antitumor compound in the crude mushroom extract [Kamasuka *et. al*., 1986].

Goro *et. al*., (1970). Was extracted polysaccharide extract from *Lentinus edodes* using hot water extraction method, and they obtained

a brown powder extract and then they purify this extract by gel filtration using DEAE-Cellulose column (3.5x30 cm). They obtained 16 fractions that exhibit antitumor activity but in different degrees.

In 2001, Yap and Ng was isolated a crude polysaccharide extract from certain edible mushrooms by ethanol precipitation and then they purify the crude extract by a combination of purification methods including gel filtration, ion-exchange chromatography and affinity chromatography.

2.3 Natural Products as Anticancer Agents

Cancer has been recognized for more than 2000 years ago, it was noticed by Hippocrates and Galen. However, Al–Zahrawi was considered as the first physician who described cancer to look a little like crab because of finger like projection [Yaseen, 1990]. Cancer cells exhibit uncontrolled and disorganized growth.

Normal cells divided only about 50 times, while cancer cells enter the cell cycle repeatedly and never differentiate. It has been shown that cancer cells produce proteolytic enzymes that allow them to invade under lying and then traveling through the blood and lymph. Cancer cells start new tumors wherever they relocate. This process called metastasis [Sylvia, 1998].

Cancer cell have abnormal nuclei (enlarged nuclear) and have abnormal number of chromosomes which may be mutated and some

parts may be duplicated and some may be deleted [Tailor and Parakarma, 1995].

In the war of cancer, and through the history of treating cancer disease, natural products played an important role in the development of cancer chemotherapy. Between 1960 and 1982 the National Cancer Institute screened around 114,000 extracts from an estimated 35,000 plant samples for anticancer activity. They initiated a new natural products program with a new *in vitro* human cancer cell line screen in 1987 and in 1991, 28000 plant samples have been collected from over 20 countries to screen for anticancer activity [Cragg *et. al*., 1993].

A recent survey showed that of the 87 approved anticancer drugs over the past ten years, 62% are of natural origin or are modeled natural products parents [Cragg *et. al*., 1997].

Among those clinically useful drugs include paclitaxel (Toxol R) [Kingston, 1993], vincristine (Oncovin R) [Gerzon, 1980], podophyllotoxin [Jardine, 1980], and camptothecin [Wall *et. al*., 1993].

These substances representing some of the most exciting new chemotherapeutic agents currently available for use in a clinical setting.Although significant progress has been made in cancer chemotherapy, current drugs are ineffective against much common cancer and often very toxic.For these reasons the discovery of new drugs effective against resistant sold tumor is an important and necessary strategy in improving chemotherapy.

Undoubtedly more efforts are needed to search for new cancer drugs with the aid of better screening methods from plants and other natural sources [Remers, 1980].

2.4 Medicinal Mushroom and Host Immune Response

The immune response is a complex serious of cellular interactions activated by the entry of foreign antigenic materials into the body, such as infectious agents and variety of macromolecules. After processing by macrophage the antigen is presented to lymphocytes, which are the major effecter cells of the immune system. Lymphocyte activation by antigen result in proliferation and transformation of lymphocytes, which lead to two types of immune system: Cell-mediated immunity and Humoral immunity [Roitt, 1988].

Since currently available anticancer agents have not compacted significantly on the survival of the majority of the patients with tumors, novel therapeutic strategies are needed. One of these therapeutic strategies is by using polysaccharide extract from edible mushroom. Medicinal mushrooms have along history of use in folk medicine [Eckhardt, 1996].

In particular, mushroom useful against cancer of stomach, esophagus, lung, breast, leukemia, gastric intestine and colorectal cancer [Liang and Wang, 1999].

The active polysaccharide extracts from edible mushroom are currently the most promising class of immunoceuticals. Without

doubt, they are capable of simultaneously stimulating all the key pathways of host immunity.

Active polysaccharide stimulates macrophage and other immune phagocytic activities *in vivo*. When mice were given charcoal intravenously, then fed polysaccharide extract, both the phagocytic activity of cells in blood and the clearance of charcoal from the circulation were significantly accelerated [Yang *et. al*., 1993]. It has been shown also that mushroom extract capable of stimulating killer cells [Mizutani *et. al*., 1991; Kariya *et. al*., 1992]. These extracts activate killer cells *in vivo*, for example, the entry of polysaccharide extract into human gastric tumor mass prior to surgery caused T-cells around the site to become tumor infiltrating and develop significantly enhanced cytotoxic "killer" activity directed at the tumor [Mizutani *et. al.,* 1984]. These extracts also stimulate the activity of natural killer cells (NK) [Mizutani *et. al*., 1991; Kariya *et. al*., 1992].

2.5 Antitumor and Modulatory Active Polysaccharide from Different Sources of Organisms

There are certain polymers that recently been shown to act as potent immunomodulating agents [Tzianabos, 2000].

Compounds that are capable of interacting with the immune system to up regulate or down regulate specific aspects of the host response can be classified as immunomodulators.

Immunomodulators affect with some agents such as dosage, route of administration, timing and frequency of administration [Tzianabos, 2000].

The type of activity of these compounds exhibit can also depend upon their mechanism.

Polysaccharide derived from a variety of different microbial genera include *Streptococcus* species, *Bacteriodes fragilis*, *Candida albicans* and *Sacharomyces cerivisiae*, have shown significant promise in the treatment of infectious diseases [Shapiro *et. al*., 1986; Garner *et. al*., 1990; Muller *et. al*., 1997; Schrager *et. al*., 1998]. Antitumor effects were another promising biopharmacological activity of polysaccharides from these sources. The earliest bacterial-derived polysaccharide reported to have antitumor activity was attributed to *Serratia marcescens* and became known as Shear's polysaccharide [Ooi and Liu, 2000]. This polysaccharide could cause extensive cytotoxic damage to Sarcoma 37 tumors, but it had serious side effect. Although many other polysaccharides from bacteria such as *Escherichia coli, Streptococcus pyogenes* (ok-432), *Proteus vulgaris, Acetobacter xylinum* and *Salmonella typhimurium* have also been reported to exhibit cytotoxicity against solid tumors [Whistler *et al*., 1976].

One of the most significant factors of many of the derived bioactive polymers form medicinal mushrooms is their role as immunomodulators.

Whilst have been reported that certain medicinal mushroom extracts have the ability to modulate key components of the immune system.

The immune system plays an important role in the body's defense against infections and tumor formation. Moreover, the body's

defense against viral attack and against spontaneously arising malignant tumor cells comprises a dynamic orchestrated interplay of innate and acquired immune responses. Innate immunity (having macrophages, neutrophils, NK and dendretic cells as gatekeepers), is regulated by chemical-messengers or cytokines and by activation of inflammatory and acute phase responses [Chihara, 1992]. The mononuclear phagocyte system (example, macrophages and monocytes), dendritic cells and certain lymphocyte (example, natural killer cells) serve a number of important roles including the recognition and destruction of abnormal cells. Stimulated macrophages and natural killer (NK) cells produce cytokines such as interferons and others that are targeted towards destroying cancer cells. These are regarded as the first line in the host defense system.
The identification of mushroom derived compounds that are capable of stimulating compounds of innate and acquired immunities may be of benefit for cancer treatment, thus these immunological activities play a governing role in host recognition, targeting and destroying unwanted tumor and cancerous cells. Induction and expression of cellular immunity in host resistance to cancer and microbial infections is representing complex interactions between antigen, macrophages, and lymphocytes [Borchers *et. al*., 1999].

The ability of bioactive polysaccharide and protein-bound polysaccharide to modulate many important immune cells may due to the structural diversity, variability of these macromolecules and unlike proteins and nucleic acid, polysaccharides contain repetitive structural

features which are polymers of monosaccharide residues join to each other by glycosidic linkages.

Therefore, this enormous potential variability in polysaccharide structure gives the necessary flexibility for the precise regulatory mechanisms of various cell-cell interactions in higher organisms [Ooi and Liu, 2000].

2-6 Mechanism of Action of Antitumor Substances

Mechanism of action of antitumor substances in cancer cells is a very complicated mechanism, and the understanding of such mechanism is not only share in the treatment of cancer patients but also it share in the production of an active and novel drugs of cancer treatment [Masuda *et. al.*, 1988]. Some of these substances have a direct effect on the tumor cells, while others have an indirect effect on these cells by inducing the immune system of patient. So it have been divided the action mechanism of such substances into two classes:

i.The Direct Effect on Tumor Cells

A drug may interfere with the role of DNA or RNA template in replication or transcription directly, by reacting with it to form a complex, or indirectly, by causing structural alterations such as strand breakage, removal of bases or formation of cross links [Gale *et. al.*, 1981]. Such drugs called intercalator and such process called intercalation, and mushroom extract is considered as DNA intercalator [Eckhardt, 1996]. Many studies have been revealed that the direct

effect of the antitumor substances is by their interaction with macromolecules biosynthesis [Umezawa *et. al*., 1983].

It have been shown that the most of these antitumor substances give their cytotoxic effect through the suppressive effect of the biosynthesis of the deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) or both of them [Haskell, 1980], for example polysaccharide extract of *illudin* mushroom has a potent inhibition ability for the DNA synthesis, some of the antibiotics that isolated from *Streptomyces* species have an inhibition ability for DNA and RNA synthesis [Haskell, 1980; Jianzhe, 1987]. Such substances which interact with DNA cancer cells induced apoptosis [Eckhardt, 1996].

ii.The Indirect Effect on Tumor Cells

Cancer is forming continuously inside the body a long the life, and when it formed the body removed it immediately by its immunological processes, and so it is demonstrating that the infection rates with tumor in children and old men were raised because their immune system dose not have an efficient capacity to face this disorder in their cells [AL-Saad *et. al*., 1990].

Mushroom polysaccharide extract is used in the treatment of some cancer cases because of their excellent capacity to increase and raise the immune activity of the patient [Ooi *et. al*., 2000]. Such extract is noticed to increase the thymus weight, immunoglobulin G (IgG) content in tumor bearing mice, promoting lymphocyte proliferation and increased the production of interleukin II and interferon [Mizuno *et. al*, 1992].

2.7 *Pleurotus* Mushroom

Pleurotus mushroom considered as wood-destroying saprophytic fungi that naturally found in moderate regions of the world [Wood and Smith, 1987].

The biological classification of *Pleurotus* mushroom is:

Division: Basidiomycetes

Subdivision: Basidiomycotina

Class: Hamenomycetes

Order: Agaricales

Family: Agaricaceae

Pleurotus mushroom is characterized by the absence of the volva and the ring in their fruiting bodies and instead of that it seems to be as like as oyster, shell, spatula or reniform.

The color of these fruiting bodies has been differing according to their kind and these colors are ranging among yellow, white, gray and brown [AL-Doori, 1996].

Spores color of this genus is generally white, in spite of that, these spores may be found in another colors according to the spore print of fungi cap and so according to this, Guzman and his company have been recording that *Pleurotus djamor* contains two types of spores and one of them white and the other is brown to pinkish color [Guzman *et. al*., 1995]. The kind of *Pleurotus* mushroom that is widely produced is *Pleurotus ostreatus*, which is known as oyster mushroom. It is grown on tree trunks as shells on short led fruits, and has a good nutritional value [Al-Doori, 1996].

Pleurotus oyster mushroom is a medicinal kind of an edible mushroom. It is grown on most hard wood, wood by-products and all agriculture wastes and its flavor as oyster like, hence the generic name is oyster mushroom.

It contains antitumor, antiviral, antibacterial, antiparasitic and anticholesterol agents [Rosenheim, 1999].

2.8 Antimicrobial Activity of Mushroom Polysaccharide

Experimental studies found that polysaccharide extract of many types of mushroom exhibit antimicrobial activity, for example its stimulating expression of IL-2 receptor on peripheral blood mounonuclear cells in patients with chronic hepatitis B. Another type of mushroom extract exhibits activity against *Actinomyces* species and *Enterococcus* species [Internet III].

The medicinal properties of mushroom have been known since ancient time.

Relatively few studies are available on the biological effect of mushroom consumption.

Antimicrobial activity of mushroom was identified by using antimicrobial tests.

Mushroom polysaccharide extracts exhibit activity against a variety of bacteria including *Staphylococcus aureus, Streptococcus spp., Salmomella enteritidis, Klebsiella pneumoniae, Escherichia coli* and *Proteus vulgaris* [Britten *et. al*., 1999].

Antifungal activity of mushroom polysaccharide extract was evident against *Candida albicans* [Turnquist, 1995].

Pleurotus mushroom (*Pleurotus ostreatus*) is widely cultivated and its polysaccharide extract has various biological activities such as antimicrobial activity [Beltran *et. al*., 1997].

2.9 Biochemical Prophage Induction Assay (BIA)

There are two screening system for detection the antitumor activity of any extract, *in vitro* screening system and *in vivo* screening system [Mirabelli *et. al*., 1985].

Because mushroom polysaccharide extract produced in a low concentration, and the upper limit quantity of antitumor extract have been tested inside the body is 400 mg / kg, so the *in vivo* system have limited uses, and the *in vitro* system of screening was used [Elespuru and White, 1983; Mirabelli *et. al*., 1985].

In general the first *in vitro* screening method was used for detection of antitumor activity was the biochemical prophage induction assay (BIA), which was used by Lein and his coworker (1962). Lein was noticed that there is a relationship between the ability of antitumor extracts to induce the phage λ in *E. coli* CI 857 (lysogen) and their activity to suppress and inhibit many kinds of tumors [Lein *et. al.*, 1962]. Endo and his coworkers (1963), and Heinemann and Haward (1964) were ensured Lein relationship.

This test include induction of phage λ present in *E. coli* CI 857 (lysogen) by the tested antitumor substance through the detection of the presence of β-galactosidase enzyme, and Elespuru and White were developed this test and become a highly specialized test for

antitumor substances that interact with DNA [Elespuru and White, 1983].

2.10 Lymphocyte Transformation

Lymphocytes are small cells about 8-10 μm in diameter, with scanty cytoplasm and spherical nucleus occupying almost the entire cell. The nucleus condensed chromatin that is strongly basophile.

Nowell, (1980) was the first scientist who used the lymphocyte transformation assay which could be defined as "a specific chain of morphological and biological changes that occur in the lymphocytes when activated by specific antigen or non specific mitogen" such as phytohaemagglutinine (PHA) that activate T-cells, pock weed mitogen (PWM) that activate both T and B-lymphocytes and lipopolysaccharide (LPS) that activate only B-lymphocytes.

Activation occur through four phases, that begin with resting or silent phase(G0), the first growth phase (G1), protein and nucleic synthesis phase (S-phase), and finally the second growth phase (G2).

The resting phase begin when the specific antigen or mitogen bind through specific legend with a receptor on the surface of the lymphoid cell (lymphocyte), which lead to the activation of the enzymes that responsible for the activity of the cell, such as Cyclic Guanidine Mono Phosphate (CGMP) and whose percentage increased during the first minutes of activation and thus lead to the appearance of the morphological changes as a result of the transformation of rest, small cells (lymphocytes) to blast cell called (lymphoblast), where there is an increase in the size of lymphocytic cell, increase in the

number of vacuoles, and the nucleic become more visible inside the cell as result of the accumulation of the nucleus protein [Stites, 1994].

On the other hand, biological changes involve increasing the cell membrane permeability, increasing in the percentage of penetration of positive ions such as K^+ to the inside of the cell, at this point the cells inter the (G1) phase, in which there is a continuous penetration of both glucose and Ca^{+2} ions, that are essential for the synthesis of other enzymes in the nucleous, this phase characterized by production of protein that are essential for the cell, ribose nucleic acid (RNA) and lymphokines , this phase occur during 12-24 hours. After that the cells inter the (S-phase), which characterized by the synthesis of deoxyribose nucleic acid (DNA) and doublication of the cellular chromatin, and it is reach its maximum point during 48hours. Finally the activated cells inter (G2) phase after 72 hours of activation with the specific antigen or mitogen [Al-Allak, 1986; Dotsik and Sanderson, 1994].

2.11 Cytotoxic assay

Cytotoxic assay is a rapid, sensitive and inexpensive method for measuring the toxic effect of any material on suspension cell culture in 96-well microtiter tissue culture plate. This test is suitable for ordinary laboratory purposes and for large-scale applications [Philip *et. al.*, 1990].

Because the importance of this assay, it was used by a number of scientists in order to evaluate the antitumor activity of mushroom polysaccharide extract.

Yhoshida (1962). was found that polysaccharide extract isolated from *Lampteromyces japonicus* is a potent antitumor agent against Ehrlich carcinoma cell-line.

In 1966 Gregory experimented the antitumor activity of crude polysaccharide extract isolated from different types of medicinal mushroom on different tumor cell-lines such as: sarcoma 180, mammary adenocarcinoma 755, and leukemia L-1210, and he gained positive inhibitory results [Gregory, 1966].

Ikegawa *et. al*., (1968) reported that the polysaccharide extract obtained from special edible mushrooms exhibited a remarkable antitumor activity against sarcoma 180 cell-line.

The polysaccharide extract from *Lentius edodes* was examined for the antitumor activity against sarcoma 180 cell-line and it exhibit a strong inhibitory effect [Chihara, 1969].

It was examined the antitumor effect of polysaccharide extract from different medicinal mushroom using *in vitro* assay and obtained a positive result [Wu, 1998].

Fujimiya *et. al*., (1999) showed a potent cytotoxic effect of crude polysaccharide extract from *Lentinus edodes* against Meth A tumor cell-line and having no effect on normal cell culture.

It was indicated that polysaccharide extract obtained from certain edible mushrooms could cause an extensive cytotoxic damage to sarcoma 37 cell-line [Ooi and Liu, 1999].

Hyang *et. al*., (2001) were examined the antitumor activity of crude polysaccharide extract isolated by hot water extraction method form *Agaricus blazei in vitro* against several tumor cell-lines such as: sarcoma 180, lymphoma U-937, hepatocellular carcinoma SNU-182,and leukemia P388, and they obtained a positive inhibitory results. They found that the most sensitive cell-line among these tumor cell-line was sarcoma 180 cell-line.

In 2002, Daba and his company were reported that the polysaccharide extract of *Pleurotus ostratus* mushroom possess a potent antitumor activity against Erlich ascites carcinoma and sarcoma 180 cell-lines [Daba *et. al*., 2002].

Materials and Methods

3.1 Equipment and Apparatus

The following equipments and apparatus were used through out the study:

Equipment	Origin
Autoclave	Tomy (Japan)
Compound microscope	Olympus (Japan)
Cooled centrifuge	M. S.E. (U. K.)
Distillator	Kent (U.K.)
Incubator	Termaks (U. K.)
Milipore filter unit	Millipore Corp (U. S. A.)
Oven	Gallenkamp (U. K.)
pH meter	Metler-Toledo (U. K.)
Shaking incubator	GFL (Germany)
Shaker water bath	Kotterman (Jermany)
Spectrophotometer 20	Aurova Instrument Ltd. UV 201 (U. K.)
ELISA reader	Organon Teknika (Microwell system) Australia)

3.2 Chemical and Biological Materials:

The following chemical and materials were used throughout the study:

Material	Company (Origin)
Acetone, ethyl ether, sulpharic acid, alcoholic α-naphthol, sodium citrate, β-mercaptoehanol, sodium phosphate, glycial acetic acid, Geimsa stain, glucose, sodium carbonate,sodium bicarbonate,benzyl pencillin,streptomycin	BDH (U.K.)
Sepharose CL-6B	Pharmasia,Fine Chemical (U.K.)
Ethanol, Trypsin,chloramphenicol	Merek (Switzerland)
Yeast extract, nutrient broth, Trypton	Biolife (Italy)
Sodium chloride, Na_2HPO_4, NaH_2PO_4, KCl, $MgSO_4.7H_2O$, K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$	Fluka (Jermany)
Hepes buffer	Flow laboratories (U.K.)

3.3 Extraction of Mushroom Polysaccharide

3.3.1 Organisms

Two kinds of mushroom isolates were used:

a) Industrial isolate:

   It was obtained from Khurbeet factory in Ramady city.

b) Wild isolate:

   It was grown in tree trunks of house gardens and enrichment soil and it was collected from Salah AL-Deen city.

3.3.2 Extraction Method [Goro *et. al*., 1970]

a) Five hundred grams of mushroom fruiting bodies were cutted into small pieces.
b) Distal water (650 ml) was added to these pieces.
c) The mixture was boiled by shaking water bath (4 rounds / min) for 2 hr., and then it filtered with sterile gauze.
d) Four volumes of 99 % ethanol were added to the filtrate, and the precipitate was collected by the cooled centrifuge (2000 rpm, 5 ºC for 10 min).
e) The precipitate was collected and washed with ethanol, acetone and finally with ethyl ether.
f) The precipitate was dried in the oven at 60 ºC for 48 hr., then the crude extract (brown powder) was obtained and stored in to the refrigerator.

3.4 Characterization of Crude Mushroom Extract

3.4.1 Molish Test

– Solutions:

-Five percent Alcoholic α-Naphthol

-Concentrated sulpharic acid.

– Procedure [Jasim, 2000]

i. Two drops of alcoholic α-Naphthol were added to the test tube that contains 1ml of crude mushroom extract(0.1g/ml D.W.).

ii. Two milliliters of concentrated H_2SO_4 was added carefully on the inner wall of the tube.

iii. The positive result was represented by appearance of purple ring formed between the extract and the acid.

3.4.2 Phenol-H_2SO_4 Test [Dubois *et. al*., 1956]

– Solutions:

- Phenol (5 %):

It was prepared by dissolving 5g of phenol in 100 ml of D. W.

- Concentrated sulpharic acid.

– Procedure

i- One milliliter of 5% phenol solution was added to 1 ml of crude mushroom extract (0.1 g / ml D.W.).

ii- Five milliliter of concentrated H_2SO_4 were added to the previouse mixture.

iii- The control was made by using D.W. (1 ml) instead of the crude mushroom extract.

iiii- Positive result was observed through changing the color of mixture to brown.

3.5 Purification of Crude Mushroom Extracts [Scopes, 1987]

3.5.1 Preparation of Gel Filtration Column Using Sepharose CL-6B

Dimensions of the column used in gel filtration were (3.5 x 35 cm)

Cylinder volume = Base area x Height

$= (\text{radius})^2 \times \Pi \times 35$

$= (3.5/2)^2 \times 3.14 \times 35$

$= 336.5\ cm^2$

Sepharose CL-6B (Pharmacia, fine chemical) was used for packing the column after degassing for 10 min., into the column which was equilibrated with the saline solution (0.2 M NaCl) for 48 hrs. at a flow rate of 2 ml / min.

3.5.2 Introduction of the Sample to Sepharose-CL Column

One gram of mushroom extract was dissolved in 5 ml the saline solution. The extract was introduced into the column by a sterilized pasture pipette gently on the column inner wall and eluted with NaCl (0.2 M) at a flow rate 2 ml / min.

One hundred fractions (containing 4 ml of wild type *Pleurotus oyster* polysaccharide extract) were collected in 10 ml test tube and the absorbency of each fraction was measured at wave length 600 nm.

3.5.3 Determination of Total Carbohydrates of Mushroom Extract Fractions

Glucose standard curve prepared according to phenol-sulpheric acid method (item 3.4.2)

The following volumes of glucose stock solution were added to test tubes (in duplicate).

Tube no.	Volume of glucose stock solution (80 mg / ml) (ml)	Volume of D. W. (ml)	Glucose concentration (mg / ml)
1	0	1.0	0
2	0.1	0.9	8
3	0.3	0.7	24
4	0.5	0.5	40
5	0.7	0.3	56
6	1.0	0	80

One milliliter of 5 % phenol solution was added for each tube, and then 5 ml of concentrated H_2SO_4 was added, shacked strongly and allowed to cool.

Optical density of each tube was read in spectrophotometer 20 at wave length 490 nm, the first tube was used as blank. The standard curve was obtained by drawing a histogramic relationship between the optical density on 490 nm and glucose concentration (figure 3-1).

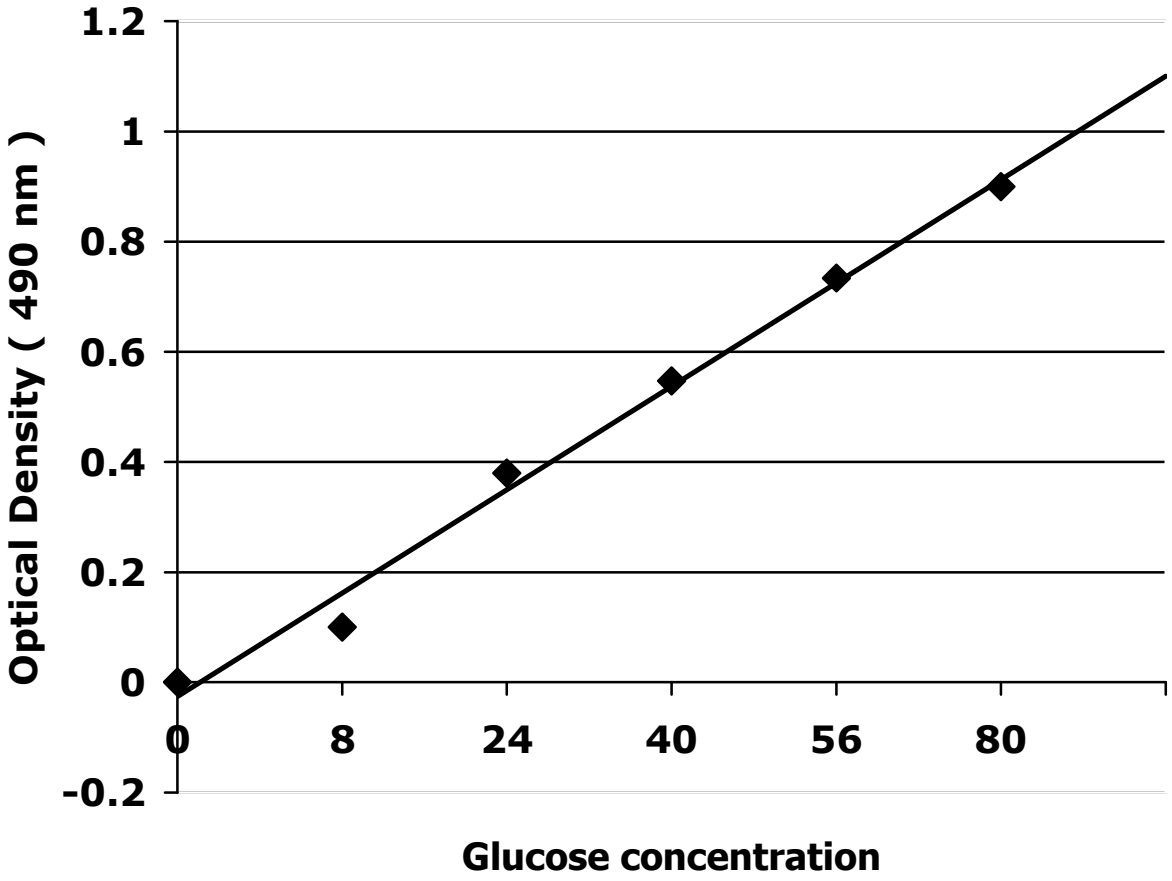

Fig (3.1) Glucose standard curve.

Carbohydrate percentage of the fractions of mushroom extract has determined by the above method and according to glucose standard curve.

3.6 Antibacterial test

3.6.1 Bacteria

Bacteria were obtained from biotechnology department which were *Staphylococcus aureus* ATCC and *Escherichia coli* ATCC.

3.6.2 Media and Solutions

a. Nutrient agar medium

The nutrient agar medium was prepared by mixing the following ingredients

Nutrient broth	8 gm
Agar	20 gm
D. W.	1000 ml

This medium was sterilized by autoclave at 121^{o}C for 15 min.

b. Mushroom Extract

It was prepared by dissolving 0.1 g of crude mushroom extract (wild type and industrial type) in 1 ml of D.W., and then sterilized by filtration through 0.22 µm Millipore filter.

3.6.3 Procedure [Sakthivel *et. al.*, 2001]

To detect the antibacterial activity of crude mushroom extract, agar-well method was used and as following:

i. Nutrient agar medium was poured into sterile petri dishes.

ii. Plates were spreaded with 100µl of overnight bacterial culture suspension using a sterile spreader.
iii. Wells (5mm diameter) were made into plates with the help of sterile cork borer.
iv. Twenty-five microliter of crude mushroom extract (wild type and industrial type) was pipetted into each well under sterile conditions.
v. The plates were incubated at 37ºC for 24 hr.
vi. Antibacterial activity of crude mushroom extract was determined by measuring the inhibition zone appeared around the wells.

3.7 Biochemical Prophage Induction Assay (BIA)

3.7.1 Bacteria

Escherichia coli CI857 (lysogen) that contain λ phage [lysogenized in β-galactosidase gene (Lac z gene)] was used.

3.7.2 Media and Solutions

a- Luria Bertani Broth (LB) [Maniatis *et . al*., 1982]

Ingredient	Quantity (g)
Tryptone	10
Yeast extract	5
NaCl	10
Glucose	1

After ingredients were dissolved in 1L of D.W., pH was adjusted to 7.0 and sterilized by autoclave at 121 ºC for 15 min.

b- ZCM Buffer

Ingredient	Quantity
Na_2HPO_4	8.5 g
NaH_2PO_4	5.5 g
KCl	0.75 g
$MgSO_4.7H_2O$	0.246 g
Chloramphenicol	0.025 g
β-mercaptoethanol	2.7 ml

After ingredients were dissolved in 1L of D.W., pH was adjusted to 7.0 and then stored in refrigerator.

c- A-medium

Ingredient	Quantity (g)
K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH_4)_2SO_4$	1 g
Sodium Citrate	0.5 g
Dissolved in 1L D. W.	

After ingredients were dissolved in 1L of D.W., pH was adjusted to 7.0 and sterilized by autoclave at 121 ºC for 15 min.

d- O-Nitrophenyl-β-D-galactopyranoside (ONPG) (substrate of β-galactosidase)

It was prepared in concentration of 2 mg / ml by mixing 0.2 g of ONPG in 100 ml of A-medium and stored in refrigerator

e- Sodium Carbonate (1 M)

It was prepared by dissolving 105.99 g of sodium carbonate in 1L of D.W.

f- Mitomycin C (10 µg / µl) (Sigma chemical compound USA)

It was prepared by dissolving 0.002 g of mitomycin C in 0.2 ml of methanol.

3.7.3 Procedure [Elespuru and White, 1983]

i. Fifty ml portion of sterilized LB medium was inoculated with a loopfull of bacterial culture (*E. coli CI857*). The inoculated medium was incubated in the shaker incubator at 37 ºC for 24 hrs, then 0.5 ml of bacterial culture have been used to inoculate another 50 ml of LB medium and incubated for 2.5-3 hrs until the optical density (O D) reached 0.4 at wavelength 600 nm.

ii. Twenty five µl portion of the mushroom extract (wild type and industrial type) (0.1 g / ml saline) was added to 0.5 ml of bacterial culture ($O. D._{600}$ = 0.4) in a sterile test tube.

iii. The positive control was made by adding 25 µl of mitomycin C to 0.5 ml of bacterial culture ($O. D._{600}$= 0.4) in a sterile test tube.

iv. The mixture was incubated in a water bath at 37 ºC with a slow shaking (50 rounds / min) for 3 hrs to ensure the prophage induction process.

v. The prophage induction process was stopped by adding 4.5 ml of cold ZCM buffer.

vi. One ml. of ONPG was added to the reacted mixture to estimate the enzymatic activity of β-galactosidase. The mixture was incubated in water bath at 28 ºC for 2 hrs

vii. The enzymatic activity was stopped by adding 2.5 ml of 1M sodium carbonate.

(In the blank tube 2.5 ml of 1M sodium carbonate was added before the ONPG was added to ensure stopped of the enzymatic activity).

viii. Optical density of samples has been estimated by spectrophotometer at wave length 420 nm.

ix. The enzymatic activity have been estimated according to the following equation: -

$$\text{Enzyme (unit / tube) quantity} = \frac{100A_{420}}{t}$$

A: Optical density of enzymatic activity.

t: Time of enzymatic activity.

3.8 Lymphocyte Transformation Assay [Al-Taee, 2003]

3.8.1 Stock Solutions:-

a- Antibiotic

Ingredient	Quantity
Benzyl penicillin	1000,000 I. U.
Streptomycin	1g
D.W.	100ml

The solution was sterilized by filtration through 0.22 μm millipore filter then dispensed into aliquots and stored in freezer (-18 - -20 ºC).

b- Hepes buffer (Flow laboratories U.K.)

(N-2-Hydroxyethyl Piperazine-N-2-Ethane Sulphonic acids). It is prepared readily with 1M.

c- Serum (Sigma chemical compound USA)

Fetal calf serum (FCS) was inactivated by heating at 56 ºC for 30 min. in a water bath, and then dispensed into aliquots 20 ml and stored in freezer (-18 - -20 ºC).

d- Sodium bicarbonate ~~td.~~

It was prepared by dissolving 4.4 mg of NAHCO3 into 100 ml D.W.,then, sterilized by the autoclave at 121ºC, for 15 min., and stored at 4ºC.

e- Tissue culture medium (pH = 7.2)

Ingredient	Quantity
RPMI-1640	10.4 g
1M hepes buffer	10 ml
Sodium bicarbonate	2 ml
Antibiotics	10 ml
Serum(FCS)	100 ml
and then complete the volume to 1000ml by adding D.W.	

After dissolving the ingredients into D.W. to obtain a total volume of 1L, it was mixed well, sterilized by filtration through 0.22 μm millipore filter, pH was adjusted to 7.2, then dispensed to 20 ml aliquots and stored in the freezer (-18- -20 ºC).

f- Phytohemagglutinine (PHA)

Crude PHA was obtained from the cancer researches center, dispensed into 2 ml aliquots, then stored in the freezer (-18- -20 ºC).

g- Hypotonic solution

Potassium chloride hypotonic solution (0.075 M) was prepared by dissolving 0.5587 g of KCL powder in 100 ml of D.W., and stored at 4ºC.

h- Fixative

The fixative used was made freshly by mixing 3 volumes of absolute methanol with 1 volume of glycial acetic acid 3:1(V / V).

i- Sorenson's buffer

It was prepared by dissolving 9.47 g of Na_2PO_4 and 9.08 of KH_2PO_4 in 100 ml D.W., pH was adjusted to 6.8, then stored at 4ºC.

j- Giemsa Stain (Sigma chemical company (USA)

It was prepared by adding 2 g of Curr R_{66} Giemsa powder to 100 ml of absolute methanol, stirred for 2 hrs. at 50 ºC on the hot plate, incubate at 37ºC for 24 hrs and filtered before use. The stain was diluted before use by mixing 1 volume of filtered stain with 4 volumes of Sorenson's buffer for 2-5 min. The stain was stored in the dark.

3.8.2 Procedure

i. Blood culturing

Three hundered microliter portion of heparinized blood was added to a sterile test tube containing 2.5 ml of RPMI 1640 tissue culture medium as control. Second tube contained 0.3 ml of heparinized blood, 2.5 ml of RPMI1640 tissue culture medium, and 50 µl mitogen (PHA). The third tube contained 0.3 ml of heparinized blood, 2.5 ml of RPMI1640 tissue culture medium, and 50 µl of wild type mushroom polysaccharide extract (0.1 g/ml saline). While the fourth contained 0.3 ml of heparinized blood, 2.5 ml of RPMI1640 tissue culture medium, 50 µl mitogen (PHA), and 50 µl wild type mushroom extract.

ii. Harvesting : -

After 72 hrs. of incubation at 37 ºC, the cell suspension was mixed gently and centrifuged at 1200 rpm for 10 min at room temperature, then the supernatant was discarded and the remaining cells pellet was resuspended in hypotonic solution.

iii. Hypotonic Treatment:-

The cells were resuspended in 2 ml prewarmed KCl (0.075 M) at 37ºC with continuous shaking. Then the volume was made up to 8 ml by adding more prewarmed KCl gradually with constant shaking. The cells suspension was then incubated at 37ºC for 90 min. with shaking. Cells were collected by centrifugation at 1200 rpm for 10 min. Supernatant was discarded and cell pellets were treated with the fixative.

iv. Fixation

A portion of 5 ml of freshly made fixative (methanol and glycial acetic acid, 3:1 V/V) was added dropwise with continuous agitation to the cells. Cell suspension was then centrifuged at 1200 rpm for 10 min. at room temperature. The fixative was decanted and another 5 ml freshly made fixative was added and the cells were collected by centrifugation. The fixative was changed three times before spreading the cells on the slides.

v. Slide Preparation

Cell suspension was pelleted by centrifugation at 1200 rpm for 10 min. The supernatant was discarded and cells resuspended in 1 ml amount of freshly made fixative, then with a pasture pipette 2-3 drops

of cell suspension were dropped with pasture pipette from 30 cm onto wet, chilled, grease fresh slide and allowed to air dry at room temperature for staining.

The remaining of cell suspension was stored at -20 ºC.

vi. Staining

Slide were stained with freshly made Giemsa stain (1 part of Giemsa stain to 4 parts of sorenson's buffer) for 5-8 min. Slides were then washed by the same buffer, allowed to air dry at room temperature and under light microscope by oil emersions lens. The numbers of lymphoblast and lymphocyte cells were counted and the lymphocyte transformation percentage was obtained according to the following equation:-

$$\text{lymphocyte transformation \%} = \frac{\text{Lymphoblast cells}}{\text{total (lymphoblast + lymphocyte cells)}} \times 100$$

3.9 Cytotoxic Assay

3.9.1 Stock Solutions

a- Antibiotics

As described in lymphocyte transformation assay (item 3.8.1)

b- Serum

As described in lymphocyte transformation assay (item 3.8.1)

c- Tissue Culture Media (pH=7.2)

As described in lymphocyte transformation assay (item 3.8.1)

The ingredients were mixed well, sterilized by filtration through 0.22µm Millipore filter, pH was adjusted to 7.2, then dispensed to 20 ml aliquots and stored at -20 °C.

d- Phosphate Buffer Saline (PBS)

NaCl	8 g
KCl	0.2 g
Na_2HPO4	1.15 g
KH_2PO4	0.2 g

After the ingredients were dissolved in 1L of D.W.The components were mixed well and then sterilized by autoclave at 121°C for 15 min.

e- Elution Solution

One volume of absolute ethanol was mixed with 9 volume of phosphate buffer saline (PBS).

f- Neutral Red Stain

One gram of neutral red stain was dissolved in 1000 ml of D.W.

g- **Trypsin**

It was prepared by dissolving 1 g of trypsin in 1000 ml of D.W.

3.9.2 Typea of Cell Cultures

A. Myloma cell-line

It was obtained from department of Biotechnology at AL-Nahrain University.

B. Chick-embryo fibro blast cells.

It was obtained by the following procedure of **Wiley, 1994**

-The Procedure

a. The eggs were incubated at 37 ^{o}C in the incubator for 11 days.

b. They were swabbed with 70 % alcohol.

c. The top of the shell was crack and the edge was peeled off of the air sac with sterile forceps.

d. The embryo was taken out with sterile curved forceps gently under the head.

e. The embryo was transferred to sterile petri dish then cutting into small pieces and then rinsed several times with PBS and the final rinse with trypsin.

f. Trypsin-PBS (1:9) was added to the pieces and left on the stirrer for 5 min.

g. The filtrate was collected in sterile universal containing some of serum to stop trypsin action.

h. The precipitate was taken and trypsin-PBS was added again and left on the stirrer for 10 min.

i. The filtrate was collected in the same universal and also trypsin-PBS was added to the precipitate and left on the stirrer for 15 min.

j. The final filtrate was collected in the same universal, and the cells were obtained by using centrifuge at 2500 rpm for 5 min.

k. Tissue culture media of RPMI-1640 was added to the precipitated cells and dispersed by using sterile pasture pipette.

3.9.3 Procedure [Abdul-Majeed, 2000]

i. Two lines were used for the crude mushroom extract (o.2 g / ml in saline) and 2 lines were used for the positive control (saline solution).

ii. The first 3 wells of each sample line were contained 50 µl of the tested sample.

iii. Two fold serial dilution were made from the third well of each line which contain 50 µl tissue culture media till the twelfth well.

iv. A 150 µl cell suspension was added to all wells of the tissue culture plate.

v. Two lines were used to make negative control in which they contain only 50 µl tissue culture media and 150 µl cell suspension.

vi. The cultured plates were incubated at 37 ºC.

vii. The culture plates were removed after 72 hr., and 50 µl of 0.01% neutral red solution was added to each well then reincubated at 37 ºC for 1hr. After incubation the medium was discarded, and the wells were eluted with the eluant solution methanol / PBS (phosphate buffer saline) (1:9) to elute the dye from the viable

cells. The viable cells will take the dye, while the dead cells will not do so.

viii. The plate was read by ELISA reader at an optical density of 492 nm.

ix. This test was done twice, in the first time chick-embryo fibroblast cell culture (normal cells) was used, and in the second time Myloma cell culture (tumor cells) was used.

References

- Abdul-Majeed, M. R. (2000). Induction and characterization of SU-99 plasmacytoma cell line and its effects on mice immune response, Ph, D., Baghdad, Iraq, College of Science, Al-Nahrain University.
- Aderson, W. A.; Moreau, P. L. and Devoret, R. (1980). Induction of prophage λ by daunorubicin and derivatives, correlation with antineoplastic activity. Mutat. Res. 77: 197-208.
- Al-Allak M. B. (1986). Response of human blood lymphocyte to chemical and physical mutagen. Fourth scientific conference Research Council. (Vol. 5), Part 2, PP: 876-888.
- Alomran A., Mohammed S. G., Sheuton B. K., Donnelly P. K., David F., George P. and Taylor R. M. R. (1984). Isolation and characterization of suppressive peptide from α-2 protease interaction. In; Electrophoresis 83. Ed. By, Hirai, H., PP. 387-390. Walter de Gruyter and Co. Berline, New York.
- Al-Taee, Maha Fakhry (2003). Correlation between the carcinoembryonic antigen level and immunostatus of gastrointestinal tract cancer's patients. Master thesis in college of Science, AL-Nahreen University.
- Balandrin, M. F.; Kinghorn, A. D.; Farnsworth, N. R. (1993). Human medicinal agents from plants Kinghorn, A. D.; Balandrin, M. F., Eds., ACS Symposium series 534(10):2-12.

- Beltran, M.; Estarron, M.; and Ogura, T. (1997). Volatile compounds secreted by the oyster mushroom (*Pleurotus ostreatus*) and their antibacterial activities.Journal-agricultural. 45(10):4049-4052.
- Bensky, D. and Gamble A. (1993). Materia medica of Chinese Herbal medicine, Eastland Press, Seattle, p.338-341.
- Britten, C. D.; Hilsenbeck, S. G.; Eckhardt, S. G.; Marty, J.; Mangold, G.; Macdonald, J.R.; Rowinsky, E. K.; Von. Hoff, D. D. and Weitman, S. (1999). Enhanced antitumor activity of 6-hydroxymethylacyl fulvene in combination with irinotecan and 5-fluorouracil in the HT29 human colon tumor xenograft model. Cancer-Res. 59(5): 1049-53.
- Bushdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Druker BJ, Lydon NB, (1996). Inhibition of the Ab1 protein-hyrosine kinase in vitro and in vivo by a 2 phenylaminopyrimidine derivative. Cancer Res. 56: 100-104.
- Carmichale. J.; Degraff, WG.; Gazder, A.; Mina, JD. and Mitchell, JB. (1987). Evaluation of tetrazolium based semi-automated colorometric assay: assessment of chemo-sensitivity testing. Cancer Res. 47: 937.
- Catino, J. J.; Francher, D. M.; Edinger, K. J. and Stringfellow, D. A. (1985). A microtiter cytotoxicity assay useful for the discovery of fermentation derived antitumor agents. Cancer Chemother. Pharmacol. 15: 240-243.

- Chang, S. T., Lau, O. W. and Cho, K. Y. (1981). The cultivation and nutritional value of Pleurotus sajor-coju. Eur. J. Appl. Microbial. Biotechnol. 12 (1): 58-62.
- Chelawat, S. and Choudhary, M. (1994). Nutrient Composition of Pleurotus Mushrooms. National symposium on mushroom, Solan, India. P. 99.
- Chihara, G. (1969). Inhibition of mouse Sarcoma 180 by Polysaccharides from *Lentinus edodes* (Shiitake). Nature, 222: 637.
- Chihara, G. (1992). Immunopharmacology of Lentinan, a polysaccharide isolated from Lentinas edodes: Its applications as a host defence potentiator. International Journal of Oriental Medicine, 17: 57-77.
- Cohen, S. S.; Flaks, J. G.; Barner, H. D.; Loeb, M. R. and Lichtenstein, J. (1958). The mode of action of 5-florouracil and its derivatives. Proc Natl. Acad. Sci. USA. 44: 1004-1010.
- Cooper, G. M., (1993). The Cancer book Jones and Bartlett Publishers, Boston :P.7.
- Corcoran, J. W. and Hahu, F. E. (1975). Antibiotics. 111. Mechanism of action of antimicrobial and antitumor agents. Spring-verlag, Berlin Proceedings of the Society for Experimental Biological Medicine, (221): 281-293.
- Cragg, G. M.; Boyd, M. R.; Cardellina II, J. H.; Grever, M. R.; Schepartz, S. A.; Sander, K. M.; Suffness, M. (1993). In Human Medicinal Agents from plants Kinghorn, A. D., Balandrin, M. F., Eds., ACS Symposium series 534(10): 81-95.

- Cragg, G. M.; Newman, D. J.; Sander, K. M., (1997). Human Medicinal Agents from plants Kinghorn. J. Nat. Prod., 60, 52-60.
- Czopandkay, J. and Kay, J. (1991). Isolation and characterization of β-glucan receptor on human mononuclear phagocytosis. *J. Exp. Med.*, 173: 1511-1520.
- Daba AS, Wissa Jwanny E, Esmat AY, Rashad M, Fattah A (2002). Antitumor activity of polysaccharides from *Pleurotus ostreatus* fruiting bodies and mycelia cultivated on date waste media. Egyptian J. Biochem. Mol. Biol. (In Press).
- Dotsik, E. N. and Sanderson G. J. (1994). A flourometric assay for determining cell growth in lymphocyte assay. J. Immunol, Methods; 105: 55-62.
- Dubois M; Gilles K.; Hamilton J.; Rebers P.; and Smith F. (1956).Colorimetric method for determination of sugars and related substances. Anal Chem. 28:350-356.
- Ebina, T.; Fujimiya, Y. (1998). Antitumor effect of peptide-glucan preparation extracted from *Agaricus blazei* in double-grafted tumor system in mice. Biotherapy.
- Eckhardt, S. G. (1996). New anticancer agents with novel mechanisms of action (Metting abstract). Non-serial. 6th International Congress on Anti-Cancer Treatment, February 6-9, 1996, Paris, France, P. 39.
- Ehrkem, M.; Reino, J.; Eppolito, C. and Mihich. E. (1983). The effect of PSK, a protein-bound polysaccharide on immune

response against allogenic antigens. International Journal of Immunopharmocolgy. 5: 35-42.

☒ Elespuru, R.; White, R. (1983). Biochemical prophage induction assay: A Rapid Test for Antitumor Agents that interaction with DNA. Cancer Research 43, 2819-2830.

☒ Endo, H.; Ishizawa, M.; Kamiya, T. and Sonoda, S. (1963). Relation between tumoricidal and prophage inducing action. Nature. 198: 258-260.

☒ Fisher M and Yang LX. (2002). Anticancer effects and mechanisms of polysaccharide-K (PSK): implications of cancer immunotherapy. Anticaner Res. 22: 1737-54.

☒ Flegg, P. (1992). Future strategies for mushroom production mushroom Res. 1 (1): 13-18.

☒ Flegg. P. (1994). Mushroom cultivation-future perspectives. National symposium on mushrooms. Solan, India, P. 50.

☒ Fujimiya, Y.; Suzuki, Y; Katakura, R.; Ebina, T. (1999). Tumor specific cytocidal and immunopotentiating effect of relatively low molecular weight products derived from the basidiomycetes. Anticancer Research, Jan. Feb; 19(1A): 113-8.

☒ Fullerton SA, Samadi AA, Tortorelis DG, Choudhury MS, Mallouh C, Tazaki H, Konno S. (2000). Induction of apoptosis in human prostatic cancer cells with beta-glucan (Maitake mushroom polysaccharide). *Mol Urol*. Spring; 4 (1): 7-13.

☒ Gale, E. F.; Cundliffe, E.; Reynolds, P. E.; Richmond, M. H.; Waring, M. J., (1981). The molecular basis of antibiotic action, 2nd Ed. John Wiley and Sons, P. 273.

☒ Garner, R. E.; Childress, A. M.; Human, L. G.; Domer, J. E. (1990). Characterization of Candida albicans mannan-induced, mannan specific delay-hypersensitivity suppressor cells. Infection and Immunity. (58): 2613-2620.

☒ Gerzon, K.; (1980). In Anticancer agents based on natural product models Cassady, J. M.; Douros, J. D.; Eds.; Academic Press, pp. 271-317.

☒ Gordon M, Bihari B, Goosby E, Gorter R, Greco M, Guralnik M, Mimura T, Rudinicki V, Wong R, Kaneko Y, (1998). A placebo-controlled trial of the immune modulator, lentinan, in HIV-positive patients: a phase I / II trial. J. Med. 29: 305-330.

☒ Goro Chihara, Junji Hamuro, Yukiko Macda, Yoshiko Arai, and Fumiko Fukuoka (1970). Fractionation and purification of polysaccharides with marked antitumor activity from edible mushroom, National Cancer Center Research Institute, Tsukiji, Tokyo, Japan. Cancer Research 30: 2776-2781, Nov. 1970.

☒ Gregory FJ (1966). Studies on antitumor substances produced by Basidiomycetes. Mycologia 58, 80-90.

☒ Guzman, G., Montoya, L. Bandala, V. M., Mata, G. and Salmones, D. (1995). Studies in the genus Pleurotus, IV. Observations on the pink forms growing in Mexico based in the inter breeding of two different strains. Mycotaxon. L III: 247-259.

☒ Hara, M.; Mokudai, T.; Kabayashi, E.; Gomi, K. and Makano, H. (1990). The kapurimycins, a new antitumor antibiotic

produced by streptomyces. Producing organism fermentation, isolation and biological activities. J. Antibiot. 43: 1513-1518.

☒ Haskell, C. M. (1980). Cancer treatment. W. B. Sanders Co., Philadelphia. pp. 67-75.

☒ Heinemann, B. and Howard, A. J. (1964). Introduction of lambda-bacteriophage in *Escherichia coli* as screening test for potential antitumor agents. Appl. Microbiol. 12: 234-239.

☒ Hoobs, Ch. (2000). Medicinal value of *Letinus edodes* (Berk.) Sing. (Agaricomycetideae). A literature review. International Journal of Medicinal Mushroom, 2, 287-302.

☒ http://store.yahoo.com/herbal-remedies-us4/virkilify.htm

☒ http://www.gmushrooms.com/health/nmh.htm

☒ http://www.mikei.com

☒ Hyang Sook; Eun Hye; Hyun Jung; Chang-Won and Sung Jool (2001). *In vitro* and *in vivo* antitumor activities of water extracts from *Agricus blazei*. Food Sci. Biotech. Vol. 10, No. 4, PP. 335-340.

☒ Ikegawa T, Nakanishi M, Uehara N, Chihara G, Fukuoka F (1968). Antitumor action of some Basidiomycetes, especially *Phellinus lintens*. Gann 59: 155-157.

☒ Internet I:

☒ Internet II:

☒ Internet III: Mabel Mei Po Yang, Zhinan Chen and Johan Shiu Lun. (1992). The anti-tumor effect of small polypeptide from Coriolns versicolor Department of physiology, University of

Hong Kong. Department of Pathology. Xijing institute of Basic Medical Science Xian. China.

☒ Internet IV: Suki Croan (1999). Bioconversion of wood wastes into gourmet and medicinal mushrooms.

☒ Internet V:

☒ Jardine, I. (1980). Anticancer agents based on natural product models Cassady, J. M.; Douros, J. D.; Eds., Academic press, pp. 319-351.

☒ Jasim, Ali Abdulkadhm (2000). Production , Purification, and Characterization of antitumor agents local sources of *Streptomyces*. Baghdad, Iraq, College of Science, Baghdad University.

☒ Jianzhe-Y, (1987). Icons of medicinal fungi from China Science Press, Beijing, P. 145.

☒ Kamasuka, T.; Momoki, Y. and Sakai, S. (1986). Antitumor activity of polysaccharide practions prepared from some strains of Basidiomycetes.

☒ Kariya, Y.; Inoue, N.; and Kihara, T. (1992). Activation of human natural killer cells by the protein-bound polysaccharide PSK independently of interferon and interleukin 2. Immunol. Lett; 31: 241-246.

☒ Kato M.; Hirose, K.; Hakozak, M.; Ohno, M.; Saito, Y.; Ixutani, R.; Noguchi, R.; Hori, Y.; Okumoto, S. Kuroda, D.; Nomura, H.; Nishimatu, S. and Ohoyanagi, H. (1995). Induction of gene expression for immunomodulating cytokines in peripheral blood mononuclear cells in response to orally administered PSK, an

immunomodulating protein-bound polysaccharide. *Cancer Immunology and Immunotherapy*. 40, 152-156.

- Kawagishi, H.; Nomura, A; Mizuno, T. and Chiba, S. (1990). Isolation and characterization of lectin from *Grifola frondosa* fruiting bodies. Bioch. Biophy. Acta. 1034: 247-252.
- Khan, S. M. and Kausar, A. G. (1981). Prospects of mushrooms culture in Pakistan. Mushroom Sci., 11 (1): 55-58.
- Kidd-PM, (2000). The use of mushroom glucans and proteoglycans in cancer treatment. Altern Med Rev. 5: 4-27.
- Kingston, D. G., (1993); In Human Medicinal Agents From Plant Kinghoru, A. D.; Balandrin, M. F., Eds., ACS symposium Series 534, pp. 138-148.
- Kobayashi H, Matsunaga K, Fujii M. (1993). PSK as a chemopreventive agent. *Cancer Epidemiol Biomarkers Prev.* May-Jun; 2 (3): 271-6.
- Kurzrock-0R, Kantarjian-HM, Druker-BJ, Talpaz-M, (2003). Philadelphia chromosome-positive leukemias: from basic mechanisms to molecular therapeutics. Ann Intern Med. 138: 819-830.
- Lei S. and Liu Z. (1992). Effect of Ganodrma polysaccharides of T-cell subpopulations and production of interleukin-2 in mixed lymphocyte response. Pharmaceutica Sinica (Chinese) 27, 331-335.
- Lein, J.; Heinemaun, B. and Gourevitch, A. (1962).Introduction of lysogenic bacteria as a method for detecting potential antitumor agents. Nature 169: 683-784.

- Liang Z and Wang X (1999). The regulation of effect of polysaccharo-peptide PSP on human peripheral hymphocyte proliferation. Advances in Research in PSP., p. 11-17.
- Maniatis, T.; Fritch, E. F. and Sambrock, J. (1982). Molecular cloning a laboratory manual. Gold spring Harbor laboratory, New York.
- Masuda, K.; Nakamura, T.; Mizota, T.; Mori, J. and Shimomura, K. (1988). Interstrand DNA-DNA and DNA- Protein cross-links by a new antitumor antibiotic, Fkq73, in L1210 cells. Cancer Res. 48:5172-5177.
- Matsunaga K, Morita I, Oguchi Y, Fujii T, Yoshikumi C, Nomoto K. (1986). Restoration of immunologic responsiveness by PSK in tumor-bearing animals [Article in Japanese]. *Gan to Kagaku Ryoho*. Dec; 13 (12): 3468-75.
- Mirabelli, C.; Bartus, H.; Bartus, J. O.; Johnson, R.; Mong, S.; Sung, C. and Crooke, S. (1985). Application of tissue culture microtiter test for the detection of cytotoxic agents from natural products. J. Antibio. 38: 758-766.
- Mizuno, T. and Kawai, M. (1992). Chemistry and biochemistry of mushroom fungi. Gakkai Shuppan Center, Tokyo, p.211-221.
- Mizuno, T.; Wasa, H.; Suzuki, C. and Ukai, N. (1992). Antitumoractive polysaccharides isolated from the fruiting body of *Hericium eyinaceum*, an. Edible and Medicinal Mushroom called Yamabushitake or houton. Biosci. Biotech. Biochem. 56: 347-348.

- Mizutani, Y.; Yoshida, O., (1991). Activation by protein-bound polysaccharide PSK (krestin) of cytotoxic lymphocytes that act on fresh autologous tumor cells and T_{24} human urinary bladder cancer. J Urol; 145: 1082-1087.
- Muller, A.; Ensely, H.; Pretus, R.; McNameee; EL Jones, E.; McLaughlia, W.; Chandley, W.; Browder, D.; Lowman; Williams, D. (1997). The application of various protic acids in the extraction of (1-3)-beta-D-glucan from *Saccharomyces cerevisiae,* Carbohydrate Research, 299: 203-208.
- Nowell P. C. (1980). Phytohemagglutinine and initiator of mitosis. J. Exp. Med., 3(6):132-137.
- O'Neill, M. J.; Lewis, J. A. (1993). Human medicinal Agents from plants Kinghorn, A. D.; Blandrin, M. F., Eds., ACS Symposium series 534, 10, PP. 48-55
- Ooi, V. E. and; Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. Current medicinal Chemistry, 7: 715-729.
- Ooi, V. E.; Fang, L. I.; Iqbal-Choudhary, M. (2000). Immunomodulation and anti-cancer activity of polysaccharide protein complexes. Immunomodulating Agents. Current-medicinal chemistry. 2000; 7 (7): 715-729.
- Ooi, VEC and; Liu F (1999). A review of pharmacological activities of mushroom polysaccharides. Int. J. Med. Mushrooms 1: 195-206.
- Philip Shehan; Ritsa Soreng; Dominic Scudiero, Anne Monks; James McMahon; David Vistica, Jonathan T.; Heidi Bokesch;

Susan Kenney; Michael R. (1990). New colorimetric cytotoxicity Assay for anticancer-Drug Screening.

- Quimio, T. H., Chang, S. T. and Royse, D. J. (1990). Technical guidelines for mushroom growing in the tropics. FAO. Plant production and protection. Paper 106, Rome, Italy.
- Rajarathnam, S. and Bano, Z. (1988). Pleurotus mushroom part 1B. Pathology, in vitro and in vivo growth requirements and world status. Critical reviews in food science and nutrition 269 (3): 243-311.
- Rambelli, A., Menini, U. G. (1985). Manual on mushroom cultivation. FAO. Plant production and protection. Paper 43: Rome, Italy.
- Remers, W. A., (1980). In anticancer agents based on natural product models Cassady, J. M.; Douros, J. D.; Eds., Academic press, pp. 131-146.
- Roitt J. (1988). Essential Immunology. 6^{th}, Blackwell Scientific Publications, Oxford, Australia.
- Rosenheim (1999). Bioconversion of wood wastes into gourmet and medicinal mushrooms. Germany.
- Sakthivel M. (2001). Antimicrobial activity of honey on bacteria isolated from wounds. Department of surgery, Department of microbiology, Government Medical College, Miraj and General Hospital, Sangli, Maharashtra, India.
- Schrager, H. J.; Alberti, C.; Cywes, B. J.; Dougherty; M. R., Wesseles, (1998). Hyaluranic acid capsule modulates M protein mediated adherence and acts as ligand for attachment of group A

Streptococcus to CD_{49} on human Kertinoctes. Journal of Clinical Investigations, (101): 1708-1716.

☒ Scopes, R. K. (1987). Protein purification, principles and practice 2nd edition. Asco trade typesetting Ltd., Hong Kong.

☒ Shapiro, M. E.; Kasper, D. F.; Zaleznik, S.; Spriggs, A. B.; Onderdonk; R. WI., Finberg, (1986). Cellular control of abscess formation: role of T-cells in the regulation of abscess formed in response to bacteroides fragilis. Journal of immunology, (137): 341-346.

☒ Shirata A; Sugaya K; Takasugi M and Moude K. (1993). Isolation and biological activity of toxic produced by Japanese strain of *Pseudomonas tolaasii*, the pathogen of bacterial rot of cultivated oyster mushroom.

☒ Sohi, H. S. (1988). Cultivation of edible mushrooms. National center for mushroom research, 1. CAE, Ectension Bulletin 1:5-8.

☒ Stellwagen, E. (1990). Gel filtration. In: method in enzymology (ed. Deutscher, M. P.) 182: 317-328.

☒ Stites, D. P. (1994). Clinical laboratory methods for detection of cellular immunity. In: Basic clinical Immunology by: Stites, D. P.; Terr, A. I. and Parslow, T. G., 8th edition, Lang Medical Publishing. U. S. A., pp: 195-214.

☒ Suelter, C. H. (1985). A practical guide to enzymology. A Wiley Interscine Publication, New York.

☒ Sylvia, S. Mader (1998). Biology, Fourth edition.

- Szymanski, J. (1996). Tender P the preparation with chlorine dioxide for preventive treatment and controlling bacterial blotch and general disinfection in cultivated mushroom houses.
- Tailor, C. R. and Parakarma, C. (1995). Concise pathology. Second edition. U. S. A.
- Tsukagoshi, S.; Furukawa, T.; Tamada, R. *et. al*., (1984). Langerhans cells and prognosis in patients with gastric carcinoma. Cancer; 59: 501-505.
- Turnquist SE (1995). Isolation partial purification and characterization of antimicrobial and antitumor protein derived from edible mushroom. Univ. of Missouri-Columbia.
- Tzianabos, A. (2000). Polysaccharide immunomodulators as therapeutic agents. Structural aspects and biologic function. Clinical Microbiology Reviews, (13): 523-533.
- Umezawa, H.; Okami, Y.; Kurasawa, S.; Ohnuki, T.; Ishizuka, M.; Takeuchi, T.; Shiio, T.; and Yugari, Y. (1983). Marina act on antitumor polysaccharide produced by marine bacteria. J. Antibiot. 36: 471-477.
- Wall, M. E.; Wani, M. C., (1993). In Human Medicinal Agents From Plant Kinghoru, A. D.; Balandrin, M. F., Eds., ACS symposium Series 534, pp. 149-169.
- Wasser-sp and Weis-AL. (1999). Therapeutic effects of substances occurring in higher Basidiomycetes mushroom: a modern perspective. Crit-Rev-Immunol. 19 (1): 65-96 1999.
- Whistler, R. L.; Bushway, A. A.; Sing, P., (1976). Advances in carbohydrate chemistry and biochemistry, 32: 235-257.

- Whitaker, J. R. (1972). Principle of enzymology for the food science. Marcel Dekker, Inc. New York.
- Wiley-Less and Sons (1994).Culture of Animal Cell, Third Edition, Department of Medical Oncology, University of Glas gow.
- Wood, D. A. and Smith, J. F. (1987). The cultivation of mushrooms (part III). The mushroom J. 189: 688-691.
- Wu, D.; Han, S. N.; Bronson, R. T.; Smith, D. E.; and Meydani, S. N. (1998). Dietary supplementation with mushroom derived protein-bound glucan enhances immune function in young and old mice. Journal of nutrition (USA). V. 128. (2) p. 139-197.
- Yamaguchi Y, Kagota S, Nakamura K, Shinozuka K, Kunitomo M. (2000). Antioxidant activity of the extracts from fruiting bodies of cultured *Cordyceps sinensis*. *Phytother Res*. Dec; 14 (8): 647-649.
- Yamazuki, M.; Yamashita, T.; Harada, T.; Nishikiori, T.; Saito, S.; Shimada, N. and Fujii, A. (1992). 44-homooligomycins A and B, a new antitumor antibiotics from *Streptomyces bottropensis*. Producing organisms fermentation, isolation, structure elucidation and biological properties. J. Antibiot. 45: 171-179.
- Yang, Q., Hu, Y.; Li, X., *et. al*., (1993). A new biological response modifier substance-PSP. In: Ynag, Q.; Kwok, C.; Eds. PSP International Symposium. Hong Kong: Fudan University Press; 56-72.

- Yap AT, Ng MLM (2001). An improved method for the isolation of Lentinan from the edible and Medicinal Shiitake mushroom, *Lentinus edodes* (Burk) Sing (Agaricomycetideae). Int. J. Medicinal Mushroom 3: 6-19.
- Yassen, N. Y. (1990). Cytogenic study on human colorectal cancer cell. Ph.D. thesis Univ. of Sheifild. In AL-Ta'q B. (2001). Cytogentic study on patients with chronic myelocytic leukemia. A thesis. Ms. D. Univ. of Saddam.
- Yoshida T.O. (1962). A tumor inhibitor in *Lampteromyces japonica* 3: 676-679.

المصادر العربية

- السعد، مها روؤف و الزبيدي، طارق (1995) علم المناعة. دار الحكمة للطباعة و النشر. بغداد.
- عبد الله عبدالكريم حسن الدوري (1996) انتاج الفطر *Pleurotus oyster* للاستهلاك البشري على المخلفات الزراعية و استعمال نواتجه للاستهلاك الحيواني.بغداد، العراق، كلية العلوم، جامعة بغداد.

Results and Discussion

4.1 Isolation of Crude Polysaccharide from *Pleurotus oyster* Mushroom:

Two kinds of mushroom isolates were used. The first isolate was obtained from Khurbeet factory in Ramady city which was the commercial isolate. The second isolate was a local one collected from house gardens and enrichment soil from Sallah AL-Deen city, it was identified as *Pleurotus oyster* mushroom according to the classification key of basidiomycetes fungi in department of plant protection College of Agriculture, University of Baghdad.

Several extraction methods were used in the isolation of polysaccharide from mushroom, like: hot water extraction method, 5% sodium hydroxide, ammonium oxalate and using acetic acid [Internet II].

Hot water extraction method was used in this study because this method is very easy to apply, their chemicals are very widely used, and finally the polysaccharide extracted by this method having a good and strongly biological activities [Internet II].

The crude polysaccharide extract was precipitate with ethanol (99%), then washed and dried in oven at 60 ºC for 48 hr, the result was a brown powder extract. It was obtained 9.43 g of crude polysaccharide extract from the wild isolate and 8.93 g of crude polysaccharide extract from the industrial isolate.

Goro *et. al*. (1970) were obtained a brown powder polysaccharide extract from *Lentinus edodes* by using hot water extraction method.

In 1984, it was found that the brown powder polysaccharide extract isolated by hot water extraction method seems to have good and strong biological activities, especially an antitumor activity [Internet II].

Several studies were reported the isolation of a brown powder polysaccharide extract from various medicinal mushrooms by using hot water extraction method [Kawagishi *et. al*., 1990; Mizuno *et. al*., 1992].

4.2 Identification of Crude Mushroom Extract:

Detection of the Carbohydrate Part in Crude Mushroom Extract:

Molish test is a general test, used in the detection of the carbohydrate part in the crude polysaccharide extract, which gives a positive result that represented by the appearance of a purple ring between the polysaccharide extract and the concentrated H_2SO_4.
This result was ensured by using phenol- H_2SO_4 test.

These results indicated that mushroom extract was mostly carbohydrates in its nature.

4.3 Purification of Crude Mushroom Extract Using Gel Filtration:

Gel filtration method that used in the purification of crude mushroom polysaccharide, was done as described by Scopes (1987), except that sepharose CL-6B was used instead of sephadex G75.

Sepharose CL-6B gel that used is very suitable gel because it is a multiuse gel, easily to be packaged into the column of purification, and its range is 10^4-10^6 which makes it very suitable for this extract.

Five millimeter of crude mushroom polysaccharide (extracted from wild type mushroom was poured into the column after washing with NaCl (0.2 M).

Fractions were collected and their optical density was estimated at a wave length 600 nm, because it has a greater absorbency at this wave length. Five peaks were obtained from the purification process as shown in figure (4-1).

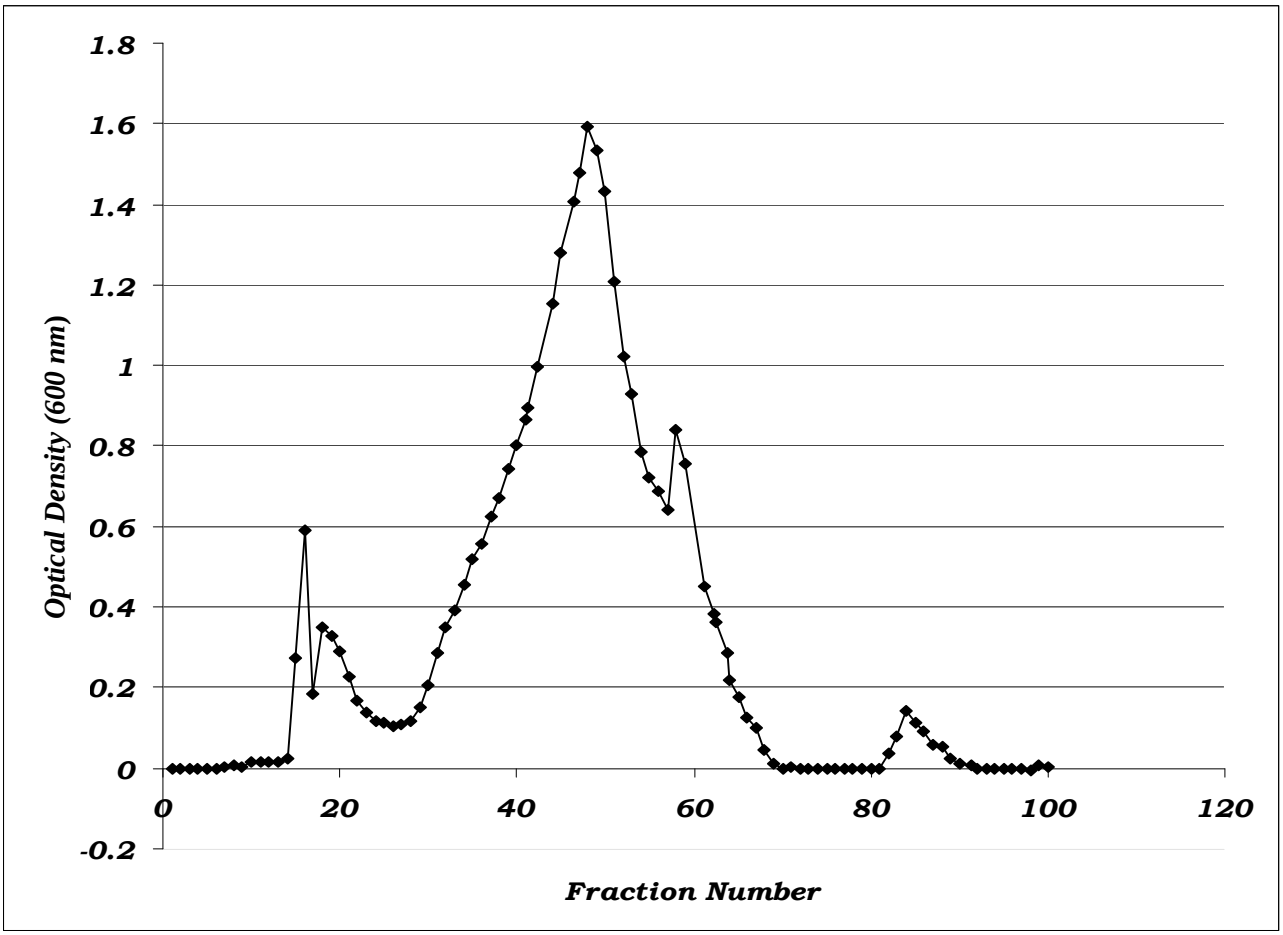

Figure (4-1) Gel filtration of local type *Pleurotus oyster* Mushroom polysaccharide extract using Sepharose CL-6B

The first, second, third, fourth and fifth peaks represented by fractions (14-17), (18-26), (27-57), (58-69) and (81-90) respectively.

The carbohydrate percentage value of each peak was estimated according to the carbohydrate standard curve (figure 3-1). They were 36, 24, 64, 49 and 15 mg/ml for the five peaks respectively.

The biological activity of purified peaks was not estimated because the crude mushroom polysaccharide extract was not stored in refrigerator so it loosed its biological activity.

In (1972) the crude mushroom polysaccharide (extracted by hot water extraction method) was purified using gel filtration in a column of purification (3.5x35 cm). The elution solution was 0.2 M of NaCl, and several peaks were obtained from this process [Internet II]. In 1986, crude polysaccharide extract of *Ganoderma lucidum* was purified and it was obtained various purified peaks that were estimated their biological activity [Internet II].

The crude polysaccharide extract of *Ganoderma tsugae* (extracted by hot water extraction method) was purified by ion-exchange chromatography, gel filtration, and affinity chromatography. Sixteen purified peaks were obtained and examined for their antitumor effect on sarcoma 180 / mice [Mizuno and Kawai, 1992].

4.4 The Antibacterial Test:

The antibacterial activity of mushroom polysaccharide (isolated from local and commercial isolates) was tested for *Staphylococcus aureus* and *Escherichia coli* by estimating the inhibition zone diameter that appears around the well that filled with the mushroom polysaccharide.

Results indicated that the crude polysaccharide extract of local isolate caused inhibition zones 0.9 cm and 1.4 cm for *Escherichia coli* and *Staphylococcus aureus* respectively. While the crude polysaccharide extract of commercial isolate caused inhibition zones of 0.8 cm and 1 cm for *Escherichia coli* and *Staphylococcus aureus* respectively.

Two expected mechanisms of action were suggested for the antibacterial activity of crude mushroom polysaccharide extract:
The first suggestion the ability of extract to affect and inhibit the DNA synthesis, and the second suggestion is the ability of it for cell-cell communication via many biochemical interactions [Corcoran *et. al*., 1975].

Hara *et. al.,* (1990) found that there is no relationship between the antimicrobial activity and antitumor activity of any substance, for example: FR-900462 antitumor agent that isolated from *Streptomyces tokashikiensis* has no antimicrobial activity. But some antitumor agents exhibit an antimicrobial activity such as 4181B and 4181A,which isolated from *Streptomyces griseus* [Yamazuki *et. al.*,1992],and also the polysaccharide extract of *Pleurotus oyster*

mushroom (extracted by hot water extraction method) exhibit an antimicrobial activity with addition to its antitumor activity [Internet IV].

Shirata *et. al.*, (1993) were found that *Pleurotus oyster* polysaccharide extract (extracted by hot water extraction method) inhibited fungal and bacterial growth, and the fungi is more sensitive to the polysaccharide extract than the bacteria.

In 1995 crude mushroom polysaccharide extract was found to contain proteins that have antibacterial activity against many bacterial strains such as: *Escherichia coli*, *Staphylococcus spp.*, *Streptococcus spp.*, *Proteus vulgaris* and *Salmonella enteritidis* [Turnqiust, 1995]. Szymanski (1996) found that crude polysaccharide extracted from mushrooms that grown on tree trunks of house gardens (wild type) has a great antibacterial activity.

Pleurotus oyster polysaccharide, extracted by hot water extraction method has a great antifungal, antiviral, antiparasitic, antibacterial and antitumor activity [Beltran *et. al.*, 1997; Internet V].

4.5 Biochemical Prophage Induction Assay for Mushroom Extract:

Biochemical prophage induction assay (BIA) is a specialized test for antitumor agents, that interact with the DNA synthesis or that affects on enzymes that interact with the DNA synthesis. This test was used for its quickiness, easy, highly sensitivity and abscene of toxic solvent like dimethyl sulfoxide. Elespuru and White were developed this test for its great important role in the screening for novel antitumor agents [Elespuru and White, 1983].

The bacteria used in this test was *Escherichia coli* CI85 (λ lysogen) that contains λ-phage inserted in its chromosome (Lac Z gene, which is responsible for the β-galactosidase enzyme production).

Two kinds of *Pleurotus oyster* crude polysaccharide (Local and commercial isolates) were used in this test.

When the *E. coli* CI 857 (λ lysogen) was incubated with crude polysaccharide extract (local and commercial isolates), this extract was caused induction to the λ-phage to jump from bacterial chromosome and then ended the inhibiting effect of λ-phage on the Lac Z gene, which lead to start β-galactosidase enzyme production.

The enzymatic activity was estimated by a quantitative method using O-Nitrophenyl-β-D-galactopyranoside (ONPG), which is the substrate of β-galactosidase enzyme.

The enzymatic activity is defined as the quantity of enzyme that needed for releasing 1 μM of ONP from ONPG in 1 min at 28 ºC.

The antitumor activity was estimated according to the enzymatic activity which determined as unit / tube.

The enzymetic activities were 12.5, 10 and 9 (unit / tube) for crude polysaccharide extracted from local isolate, crude polysaccharide extracted from commercial isolate and the positive control (mitomycin C) respectively. Such results mean that both polysaccharides extracts exhibited antitumor activity and that antitumor activity of polysaccharide extract of local isolate was more than that of the commercial isolate. According to this result, crude polysaccharide of local type isolate was selected to examined for antitumor activity by using cytotoxic and lymphocyte transformation assays.

Effect of polysaccharide extract may be excreted on the enzyme that interacting with the DNA synthesis process or affected on the DNA synthesis process directly [Cohen *et. al.*, 1958; Aderson *et. al.*, 1980].

4.6 Lymphocyte Transformation:

The results of lymphocyte transformation assay for crude polysaccharide of local *Pleurotus oyster* isolate are demonstrated in table (4-1).

The percentage of transformed cells in PHA group (slides) was less than that for group of crude polysaccharide only, while percentage of transformed cells in the group of PHA and crude polysaccharide, mixture was increased. Same table indicated that crude polysaccharide extract promoted increase in the lymphocyte

transformation process. This is means that this extract possessed immunopotentiating activities.

Table (4-1) The effect of crude polysaccharide extract of wild type mushroom on lymphocyte transformation.

	Control			**Control + PHA**			**Extract**			**Extract +PHA**		
No. of Sample	Transformed Cells		Normal cells	Transformed Cells		Normal cells	Transformed Cells		Normal cells	Transformed Cells		Normal cells
	No.	**%**	**No.**	**No.**	**%**	**No.**	**No.**	**%**	**No.**	**No.**	**%**	**No.**
1.	**91**	**35.2**	**167**	**125**	**53.6**	**108**	**138**	**57.2**	**103**	**125**	**61.8**	**77**
2.	**87**	**35.9**	**155**	**133**	**57**	**100**	**142**	**59.4**	**97**	**121**	**64.2**	**73**
Mean		**35.5**			**55**			**58**			**63.5**	

Czopandkay and Kay (1991) were shown that the immunomodulatory properties derived from medicinal mushroom is related to its backbone structure, and there is a receptor for leukocytes which, may be responsible for all reported functions of mushroom polysaccharide extract (*in vivo* and *in vitro*). Crude polysaccharide of *Ganoderma* mushroom was shown to enhance B-cell functions in both normal and tumor bearing mice [Lei and Liu, 1992].

Kato *et. al.*, (1995) reported that mushroom polysaccharide (extracted by hot water extraction method) induce gene expression of some cytokines such as:tumor necrosis factor-α (TNF-α), interleukine 1, 8 and 6 (IL-1, IL-8 and IL-6) *in vivo* and *in vitro*.

These cytokines produced by monoctyes, macrophages and other cell types mediate multiple biological effects against tumors by

enhancement of antibody production by B-lymphocytes and induction IL-2 receptor expression T-lymphocytes.

It was stated that high molecular weight and a wide solubility range of mushroom polysaccharide extract were shown to be large enough to cross-link membrane of neutrophils and monocytes that trigger cytokine release [Hobbs, 2000].

Fisher and Yang (2002) found that mushroom polysaccharide extract contains protein in its structure which boosts the immune cell production.

4.7 Cytotoxic Effect of Crude Polysaccharide Extract:

Cytotoxic assay is an important and highly selective mean that allows determining the cytotoxic effect of many agents, (in various dilutions), on different types of normal and tumor cell cultures [Carmichale *et. al*., 1987].

Two kinds of cell cultures were used in this study to determine the cytotoxic effect of wild type (*Pleurotus oyster*) crude polysaccharide. The first cell culture used was chick-embryo fibroblast cells, which represented a normal cell culture. Results (Fig 4-2) showed that the crude polysaccharide in concentrations of (10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 mg / 50 μL) showed no cytotoxic or inhibition effect on the growth of chick-embryo fibroblast cell culture, but, in contrast it showed a stimulating effect on their growth in compared with the control. However, when the dilution of crude polysaccharide extract was increased the stimulating effect decreased.

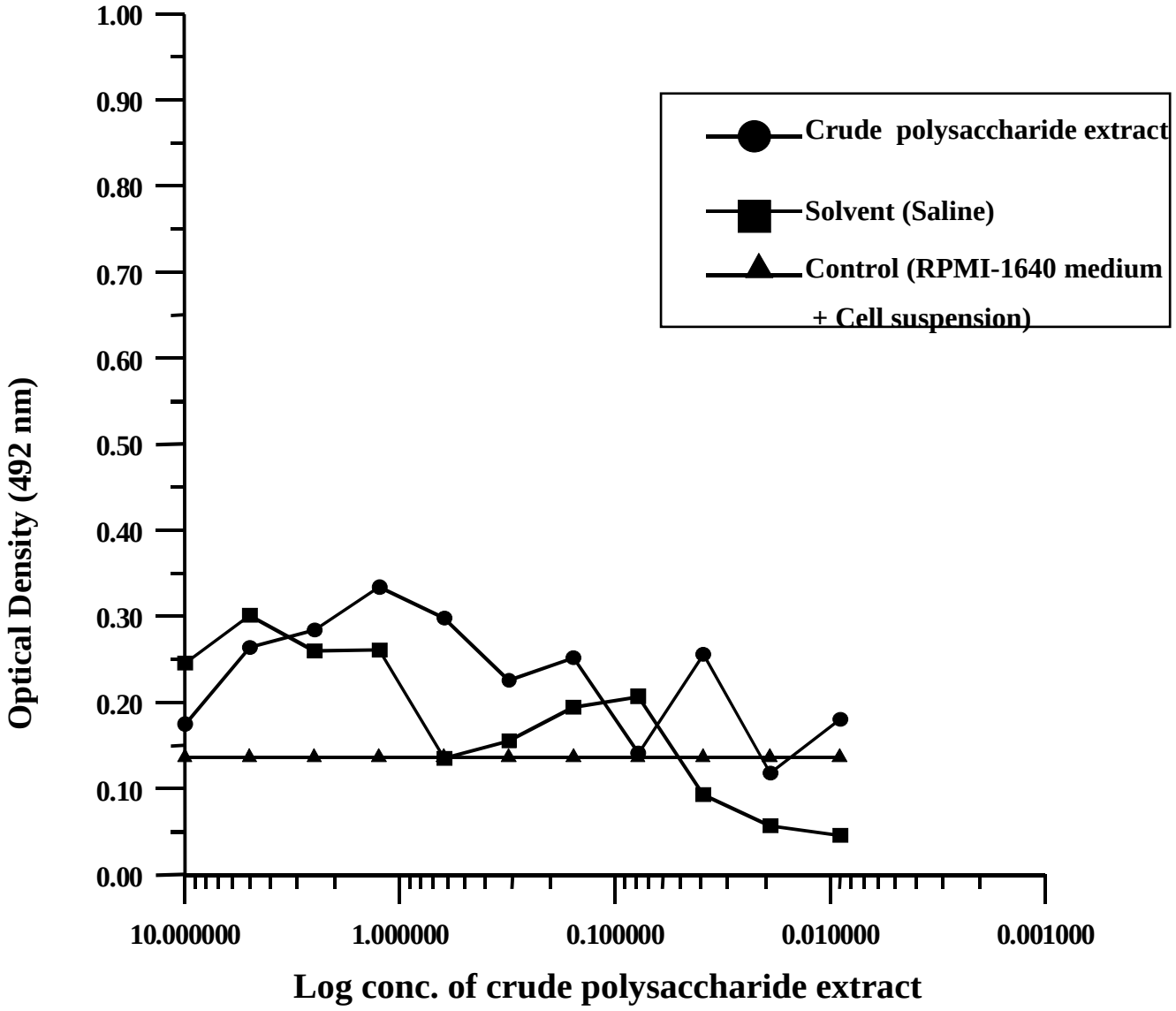

Figure (4-2) Cytotoxic effect of wild *Pleurotus oyster* crude polysaccharide on chick-embryo fibroblast cell

Many papers found that mushroom polysaccharide extract has no inhibition or cytotoxic effect on normal cells [Ehrkem *et. al*., 1983; Tsukagoshi *et. al*., 1984].

Several mechanisms of action of mushroom polysaccharide extract on normal cell culture were reported. One of these mechanisms suggested that this polysaccharide extract is consisted of macromolecules, which are unlike proteins and nucleic acid by their containing of repetitive structural features which are polymers of monosaccharide residues able to join to each other by glycosidic linkages.

This feature offers the highest capacity for carrying divers of biological functions because they have great potential for structural variability. Such enormous potential variability in polysaccharide structure gives the necessary flexibility for the precise regulatory mechanism of various cell-cell interactions [Ooi and Liu, 2000].

Another mechanism of action demonstrated that this polysaccharide extract has an effect on the tumor suppressor genes and oncogenes productions, so it regulates the normal cell growth and differentiation [Internet IV].

The second cell line used in this study to determine the cytotoxic effect of wild type (*Pleurotus oyster*) crude polysaccharide extract was myloma cell-line. Results (figure 4-3) show that the concentrations (10, 5, 2.5) mg / 50 µL of crude polysaccharide has great inhibition and cytotoxic effect against this tumor cell-line when compared with the control, whereas no significant inhibition effect

was detected by the low concentrations (0.078, 0.039, 0.019, 0.009) mg / 50 μL. Same figure also showed a little effect for the solvent (polysaccharide extract is dissolved in saline) on myloma cell line. This effect could be attributed to the permeability of these cells since myloma cell-line is a tumor cell-line, so their function and properties have many defects and one of these defects is their permeability.

Many studies indicated that mushroom polysaccharide extract has a potent antitumor activity on certain tumor and with no side effects [Whistler *et. al*., 1976]. Suki (1999) was found that the polysaccharide extract of *Pleurotus oyster* mushroom extracted by hot water extraction method has a great tumoricidal effect *in vivo* and *in vitro* [Internet V]. It was found that crude *Maitake* mushroom extract has cytotoxic effect on prostatic cancer cells *in vitro* leading to apoptosis [Fullerton *et. al*., 2000; Catino *et. al*., 1985].

Yamaguchi *et. al*. (2000) indicated the antitumor activity of crude polysaccharide extracted from *Cordyceps sinensic* mushroom.

Several explanations about the antitumor mechanism of mushroom polysaccharide extract were stated. One of these explanations is that the polysaccharide extract has an effect that inhibited DNA synthesis of tumor cells by affecting on the DNA topoisomerase enzymes (I and II) [Gale *et. al*., 1981].

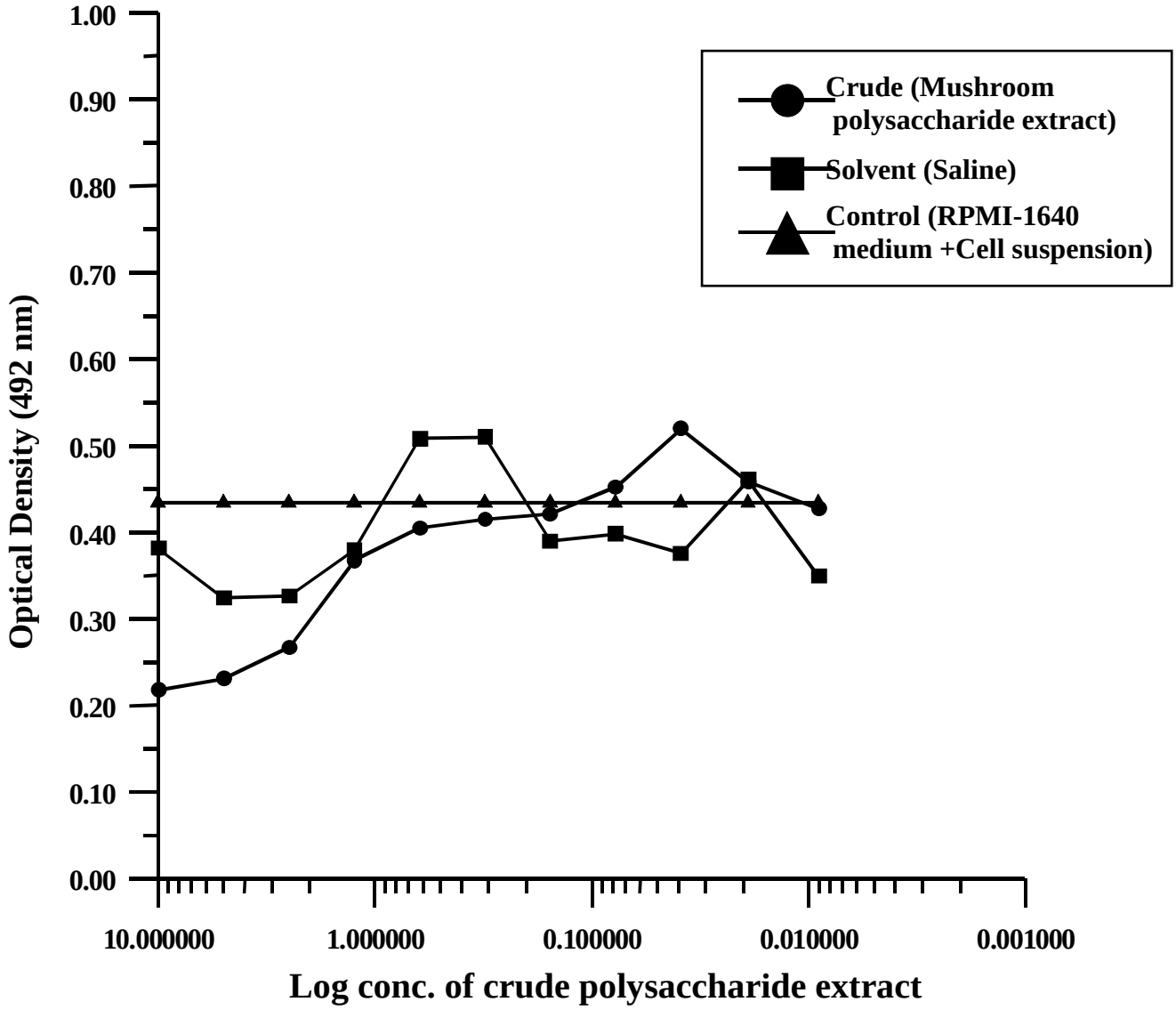

Figure (4-3) Cytotoxic effect of wild *Pleurotus oyster* crude polysaccharide on myloma cell-line.

In a study performed for testing the activity of the crude *Coriolus* mushroom polysaccharide extract, Matsunaga *et. al*. (1986) found that this extract had supporting activity for normal cell culture and cytotoxic effect on tumor cell-line by increasing the tumor necrosis factor (TNF).

Kobayashi *et. al*. (1993) were ensured the result obtained by Matsunaga in 1986.

Takema (1991) demonstrated that mushroom polysaccharide has a mechanism of action affect in tumor cells mitochondria [Internet IV].

It was found that mushroom polysaccharide extract affects the gene expression of some cytokines like tumor necrosis factor-α (TNF-α), interleukine 1, 6 and 8 (IL-1, IL-6 and IL-8), and alsot strongly affects interferon production and it induced apoptosis [Kato *et. al*., 1995].

Recent studies indicated that mushroom polysaccharide extract exhibit a tumoricidal effect by inducing T-cell production that recognize this extract as an antigen and kills tumor cells in an antigen specific manner [Kidd, 2000].

Result of cytotoxic assay supported those of biochemical prophage induction assay (BIA) which indicated that crude polysaccharide extract of wild type *Pleurotus oyster* exhibits an antitumor activity, and the most expectable mechanism of action is the inhibitoy effect of crude polysaccharide extract on DNA synthesis of tumor cells [Gale *et. al*., 1981].

Treatment of cancer with mushroom polysaccharide extract has no side effects when compared with other cancer treatments such as chemotherapy (that affects follicular hair cells and the immune cells leading to decrease the body's general defense mechanisms). It appears that this type of treatment is the promising treatment of cancer [Buchdunger *et. al*., 1996; Kurzrock *et. al*., 2003].

Summery

Two kinds of *Pleurotus oyster* mushroom isolates were collected. The first isolate was the local type and the second isolate was the commercial type.

Crude polysaccharide extract was isolated from *Pleurotus oyster* mushroom as a brown powder extract by using hot water extraction method.

This crude extract was characterized by using molish test and phenol-sulphuric test. It was found that this extract mostly carbohydrate in nature.

The crude polysaccharide extract was tested for its antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

Results indicated that crude polysaccharide extract of local isolate caused an inhibition zone of about 1.4 cm for *Staphylococcus aureus* and *Escherichia coli* was inhibited at about 0.9 cm, while the crude polysaccharide extract of industrial isolate caused an inhibition zone of about 1 cm for *Staphylococcus aureus* and *Escherichia coli* was inhibited at about 0.8 cm.

In order to determine the antitumor activity of crude polysaccharide extract, biochemical prophage induction assay was done for the crude extract of local and commercial isolates.

It was shown that the crude polysaccharide extract of local and commercial isolate capable of induced the λ-phage, that inserted in the bacterial chromosome of *E. coli* CI857 (lysogen), to jump from its place, so made the bacteria to reproduce the β-galactosidase enzyme. And the crude polysaccharide extract of local isolate gave an

enzymatic activity more than that of commercial isolate, that is mean the crude polysaccharide extract of local isolate has an antitumor activity more than that of commercial isolate. So the crude polysaccharide extract of local *Pleurotus oyster* mushroom was used in transformation and cytotoxic assays to estimate its immunomodulatory effect and antitumor activity.

Results of transformation assay indicated that the crude polysaccharide extract gave an immunomodulatory effect more than that of phytohaemagglutinine (PHA).

Results of cytotoxic assay, revealed that the crude polysaccharide extract in concentrations (10, 5 and 2.5) mg / 50 µl has a great cytotoxic effect on myloma cell line (which represented the tumor cell line), and no cytotoxic effect on chick-embryo fibroblast cell culture (which represented the normal cell culture) in concentrations (10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156) mg / 50 µl was detected.

The crude polysaccharide extract of wild isolate (*Pleurotus oyster*) was purified using gel filtration method. The purification process was done to determine which fraction was affective as antitumor, but this step was not conducted.

الخلاصة

جمعت عزلتين من فطر المشروم *Pleurotus oyster*، الاولى برية والثانية صناعية. استخلصت عزلتي المشروم مائيا ثم جففت الخلاصتان وتم الحصول على مسحوق بني اللون ،وقد تم الكشف عن الطبيعة الكربوهيدراتية باستخدام فحص الفينول-حامض الكبريتيك المركز.

اختبرت فعالية المتخلص الخام لعزلتي المشروم (البرية والصناعية) ضد نوعين من البكتريا (*E. coli* و*Staphylococcus aureus*)، اوضحت النتائج ان للمستخلصين فعالية ضد بكتيرية واضحة، اذ ادى المستخلص الخام للعزلة البرية الى تثبيط نموكل من بكتريا *E. coli* و *Staphylococcus aureus* بقطري 0.9 سم و1.4 سم على التوالي. بينما ادى المستخلص الخام للعزلة الصناعية الى تثبيط نمو بكتريا *E. coli* و*Staphylococcus aureus* بقطري 0.8 سم و1 سم على التوالي.

استخدم الفحص الكيموحيوي لحث العاثي الاولي لاختبار الفعالية المضادة للاورام للمستخلص الخام للعزلة البرية والصناعية لفطر *Pleurotus oyster*. اظهرت النتائج ان للمستخلصين قابلية على حث العاثي الاولي المغروس في كروموسوم بكتريا *E. coli* CI857 للخروج من مكانه وبالتالي اعادة قدرة البكتريا على انتاج انزيم البيتاكالكتوسايديز. و كانت قدرة المستخلص الخام للعزلة البرية على حث العاثي اكبر من قدرة المستخلص الخام للعزلة الصناعية، اذ كانت الفعالية الانزيمية باستخدام مستخلص العزلة البرية اكثر منها في حالة استخدام مستخلص العزلة الصناعية. لذا فقد استخدم المستخلص الخام للعزلة البرية في اجراء التجارب اللاحقة وهي فحوصات التحول و السمية الخلوية، ولاختبار قدرته على التحفيز المناعي ولتاكيد فعاليته المضادة للاورام.

اوضحت نتائج التحول ان للمستخلص الخام للعزلة البرية لفطر *Pleurotus oyster* فعالية ملحوضة في التحفيز المناعي اكثر من مادة phytohaemagglutinine (PHA) المعروفة بتحفيزها المناعي. اما نتائج فحص السمية الخلوية فقد بينت ان لهذا المستخلص وفي التراكيز(0.312، و0.625، و1.25، و2.5،5، و10)ملغم / 50 مايكروليتر فعالية جيدة في تثبيط نمو خلايا Myloma (الممثلة للخلايا السرطانية)، بينما كان له تاثير منشط لنمو خلايا Chick-emberyo (الممثلة للخلايا الطبيعية).

امكن الحصول على خمس قمم لدى تنقية المستخلص الخام للعزلة البرية بالترشيح الهلامي، بهدف تحديد اي جزء من المستخلص هو الحاوي على الفعالية البايوليوجية الا ان هذا العمل لم يكتمل.

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In vitro Study of Biological Activity of *Pleurotus oyster* Extract

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Pleurotus oyster

رسالة

مقدمة إلى كلية العلوم في جامعة النهرين وهي جزء من متطلبات نيل درجة ماجستير في التقانة الاحيائية

من قبل

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