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Effect of Partially Purified *Arthrobacter* spp. Polysaccharide on Nucleosome of HepG2 Cell Line

A Thesis

Submitted to the College of Science at Al-Nahrain University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

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Dedication

To my love Father and Mother

To my Brothers and Sisters

To all my colleagues and friends especially my close friends (Ahmed Shawqi, Tameem Mohammed, Ali, Mohammed Latef , Kareem and Ibrahim)

To all persons who are the reason beyond all the good things in my life, and the Ones whom I will never be able to pay back.

Nawar Bahaa

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Summary

This study was designed to investigate the effect of Gram positive bacterial polysaccharide extracted from *Arthrobacter* on the DNA banding pattern of tumor cell line in *vitro*. Ten soil samples were collected from different spots in Baghdad, the soil spots from which the samples taken were characterized from being wet, partly shade and were mixed or covered with dead plant leaves and stems. According to morphological and biochemical tests, results showed that only one bacterial isolate confirmed to belong to the genus *Arthrobacter*.

The polysaccharide was extracted from *Arthrobacter* using a method combination between the application of pressure and hot water with a recovery yield of 181mg. Chemical analysis showed that the carbohydrate and protein contents were 15.9% and 1.9% respectively. Partial purification of polysaccharide was applied by using sepharose Cl-6B gel chromatography and after purification two peaks were obtained. Chemical characterization involving the estimation of carbohydrate and protein contents showed that the first peak contained the higher carbohydrate contents (30.1%) with low protein constituents (5.7%).

The effect of *Arthrobacter* B1 polysaccharide on tumor cells was examined by studying whether bacterial polysaccharide participate in affecting the structure of tumor cell's DNA or not. HepG2 tumor cells were subjected to three treatments, first were treated with colchicine at a concentration (50µg/ml), second were treated with the partially purified polysaccharide at a concentration (1mg/ml), and the third group were not treated and used as control. Both treated and untreated cells were subjected to MNase digestion, followed by DNA extraction, electrophoresis and scanning the DNA banding pattern using gel documentation system.

Summary

Results indicated that only intact nucleosomal DNA bands were separated on the gel for the untreated cells as compared with the banding pattern of the colchicine treated DNA, in which besides to intact nucleosomal DNA, mononucleosomes, dinucleosomes and oligonucleosomes were separated on the gel following electrophoresis process. Such pattern does not affected by the gradual increase in MNase concentration. On the other hand, the DNA banding pattern for the cells treated with *Arthrobacter* B1 polysaccharide showed significant effect by the separation of nucleosomal DNA, in which mononucleosomes, dinucleosomes and oligonucleosomes were clearly separated from intact nucleosomal DNA, and such effect was markedly appeared by increasing the concentration of MNase as compared with the cells treated colchicine.

List of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
DNA	Deoxyribo Nucleic Acid.
EDTA	Ethylene Diamine Tetra Acetic Acid.
LB	Luria-Bertani.
RPMI-1640	Rosswell Park Memorial Institute- 1640.
SDS	Sodium Dodecyl Sulphate.
Tris-Hcl	Hydroxy methyl amino methane hydrochloric acid.
RPM	Round per minute.
MDR	Multidrug resistance

Chapter One
Introduction and Literature Review

1. Introduction and Literature Review

1.1 Introduction

The ability of biological materials or synthetic compounds to affect the genetic material has been well established (Wu *et al.*, 2006). Many studies appeared that bacterial preparations such as whole cells or cell wall possess marked inhibitory activity against progression and the development of tumor cells *in vitro* and *in vivo* (Thamm *et al.*, 2005; Al-Saffar, 2010). However, few studies revealed how bacterial cell wall preparation can affect the genetic materials of tumor cells.

Histones order eukaryotic DNA into structural units called nucleosomes, and histones consider being the major constituent of chromatin which are subjected to several different structural modification (Dillon *et al.,* 2004). The chromatin structure classified into euchromatin which lightly packed and relatively transcriptionally active and hetrochromatin, which is the packed and transcriptionally poor form of chromatin (Rosenfeld *et al.,* 2009).

Structural modification of chromatin plays an important role in regulating the mechanism of gene expression converting euchromatin into more open and transcriptionally active (Yamanaka *et al*, 2008).

Microbial polysaccharides may contributed in microbial such modification. Studies indicated that microbial polysaccharides were involved in several cellular processes such as molecular recognition, cell development, exert antioxidant, antiviral and antitumor activity (Shi et al., 2007).

Arthrobacter is a genus containing species of Gram positive bacteria, and its polysaccharides a represent a class of high-value polymers, that can interact with living cells displaying biological properties

such as antioxidant, immunostimulant (Liu *et al*., 2010), and the ability to inhibit tumor growth and enhances immune function (Sun *et al.*, 2009). Biological activity of the polysaccharides has attracted more attention recently in the biochemical and medical areas because of their immunomodulatory and antitumor effects. The ability of bioactive polysaccharides and polysaccharide bound proteins to modulate so many important immune functions may be due to the structural diversity and variability of these macromolecules.

According to the above mentioned information, this study was aimed to investigation whether the *Arthrobacter* polysaccharides possess a role in DNA structural modification of tumor cells. To achieve such goal, the following were designed to be performed:

1. Isolation and identification of *Arthrobacter* spp.
2. Isolation and partial purification of *Arthrobacter* polysaccharide
3. In *vitro* application of polysaccharide on HepG2 tumor cell line

1.2 Literature Review

1.2.1 Cancer

1.2.1.1 Definition

The term cancer, which mean "crab" in latin, was coined by Hippocrate in the fifth century BC to describe this disease. Although cancer has affected human since earliest time and was rare disease till the zoth century, now it's difficult to imagine that no one heard about cancer (Kleinsmith *et al.,* 2006).

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells due to a loss of regulation (Prescott *et al.*, 2002)

One defining feature of cancer is a rapid creation of abnormal cells that grow beyond their usual boundaries, and invade adjoining parts of the body and speared to other organs by a process called metastases which eventually cause death (American Cancer Society, 2008). Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths in 2008 (WHO, 2013).

1.2.1.2 Molecular Basis of Cancer

Cancer is fundamentally a genetic disease of somatic cells. Tumor karyotypes are rarely normal, and most show multiple abnormalities of both chromosomal number and structure (Knudson, 2002). Palacios *et al* (2008) pointed out that the evolution from normal cell to a metastatic cancer requires multiple genetic and epigenetic changes. Overall, there are at least three mechanisms allowing these changes to involve in the malignant transformation: an inherited mutation, a somatic mutation and methylation of the cells DNA.

1.2.1.3 Genes Involve in Cancer:

1.2.1.3.1 Oncogenes

An oncogenes is a gene that potential to cause cancer. In tumor cell, they are often mutated or expressed at high levels (Wilbur, 2009). Proto oncogene is a normal gene that can become an oncogene due to mutation or increased expression. The resultant protein is called oncoprotein (Chial *et al.,* 2006).

Proto oncogene codes for protein that help to regulate normal cell growth and differentiation. Triggering proto-oncogenes into oncogenes, will derive excessive cell division and the accumulation of cells that can form tumor (Vogelstein and Kinzler, 2004). Mendelsohn *et al* (2001) declared that Proto-oncogenes could be converted into oncogenes either by viruses (mainly RNA retroviruses) (Fig.1-1) or by somatic mutations. The activating mutations involved base substitution, amplification in gene copy number and chromosomal translocation. Croce (2008) found that the *ras* gene is a proto-oncogene that encodes an intracellular signal protein, the mutant *rasD* gene derived from *ras* is an oncogene, whose encoded oncoprotein provides an excessive or uncontrolled growth–promoting signal.

1.2.1.3.2 Tumor-Suppressor Genes

Tumor-suppressor genes, are genes that protects a cell from one step on the path of cancer (Sigal and Rotter, 2000). When this gene is mutated to cause a loss or reduction in its function, the cell can progress cancer.

Lodish *et al* (2000) suggested five board classes of proteins that are generally recognized as being encoded by the tumor suppressor genes:

a) Intracellular proteins, such as p16 cyclin-kinase inhibitor, that regulates or inhibits progression through specific stage of cell cycle.

b) Receptors for secreted hormones (e.g., tumor derived growth factor β) that function to inhibit cell proliferation.

c) Check point-control proteins that arrest the cell cycle if DNA is damaged or chromosomes are abnormal.

d) Proteins that promote apoptosis (e.g., p53).

e) Enzymes that participate in DNA repair.

The *p53* tumor-suppressor gene is functionally inactivated in 70% of human tumors; p53 can function as a transcription factor involved in either cell growth arrest or apoptosis (Lodish *et al*., 2000; Gasco, *et al*., 2002).

1.2.1.3.3 Cell- Death Genes

During the normal process of development, certain cells are supposed to be eliminated by natural program of cell death. Abnormalities in programmed cell death have been associated with a broad variety of human disease, mainly cancer. The best known example of programmed cell death failure is the sporadic form of cancer of the white blood cells known as B-cell leukemia. These cells carry a major alteration in *BCL2* cell-death gene (Michor *et al*.; 2004).

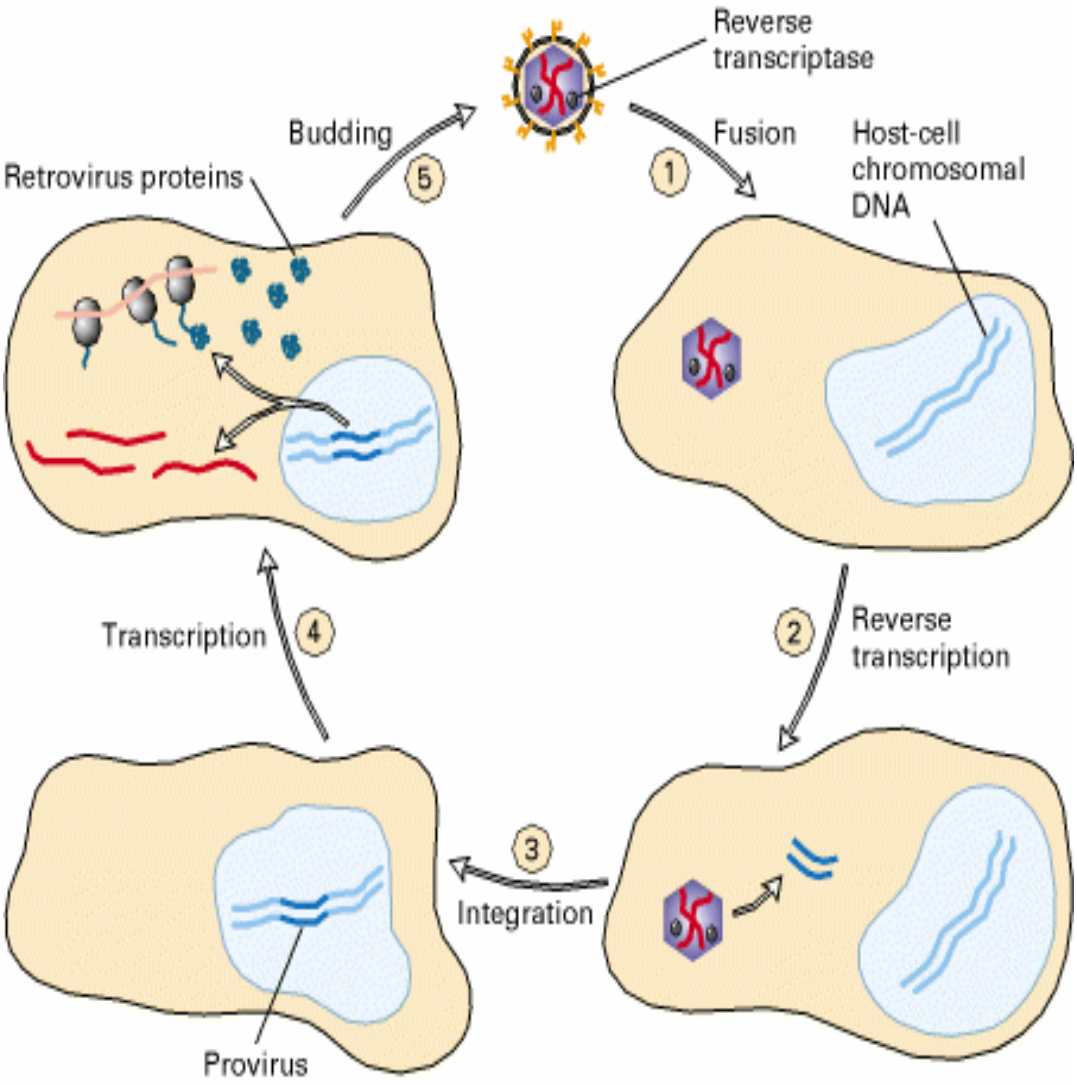

Fig. (1-1): The life cycle of retroviruses in mammalian cells (Mendelsohn *et al*., 2001).

1.2.1.4 Tumor development and chromosomal changes:

Bernstein *et al* (2007) cell specification gene expression is regulated through a vast suite of epigenetic markers, such as chromatin modification and DNA accessibility. Both considered as an important factors that regulate gene expression and depending on their conformation. Dillon

(2004) classified chromatins into: heterochromatin, which transcriptionally less active and transcriptional active chromatin.

However, some regions of heterochromatin called facultative heterochromatin, can display a transition to more open transcriptionally active conformation which characterized by its higher accessibility to DNase and micrococcal nuclease (Maison and Al mouzni, 2004).

The development and progression of tumors is derived by genetic and epigenetic changes, however the role of chromatin structure in malignant transformation still poorly understood (Druliner *et al.*, 2013). Few studies revealed that subunits of the chromatin remodeler SWI/SNF complex can act as tumor suppressors in cancer; specifically, loss of BRG1 and BRM expression has been reported as an indicator of poor prognosis in cell lung cancer (Neely *et al.;* 2002).

Nucleosome positioning regulates gene expression by modulating the accessibility of DNA to transcriptional machinery (Segal and Widom, 2009). In tumor, significant changes in nucleosome positioning result in an aberrant compaction of chromatin structure and hence altered gene expression (Wilson and Roberts, 2011).

Saeed *et al* (2011) indicating that high DNA accessibility that result from poor nucleosome occupancy has been suggested as one of the important reasons that leading to the expression of non essential genes or genes that may involve in tumor progression.

1.2.2 MNase

Micrococcal nuclease (MNase) assays of chromatin are relatively simple procedures for obtaining information about the locations of nucleosomes along DNA strands. When nuclei or permeabilized cells are exposed to MNase in the presence of a divalent cation, the enzyme makes double-stranded cuts between nucleosome particles. Treatment of

chromatin substrates with very high concentrations of MNase gives rise mostly to mononucleosome-length DNA, whereas low concentrations of the enzyme will produce one double-stranded cut every 10 to 50 nucleosomes (depending on the exact concentration). MNase can also make single-stranded DNA cuts on the histone octamer, and thus experiments to map the positions of nucleosomes are usually performed with native double-stranded DNA (John and Sons 2005).

1.2.3 Tumor Cell Line

Masters (2000) pointed out that cell culture has become an indispensable technology in many branches of the life science .It provides the basis for studying the regulation of cell proliferation , differentiation and product formation in carefully controlled conditions.

Tumor cell lines have constituted an accessible, easily usable set of biological models with which to investigate cancer biology and to explore the potential efficacy of antitumor drugs (Nayak and Dillman, 1991). Tom *et al* (1976) added that tumor cell lines provide cells with uniform origin and a source of sufficient quantity of tumor cells with pure culture population not mingled with fibroblast or epithelial cells and free of bacterial contamination.

However, tumor cell line establishment may subject to many difficulties as long as numerous unsuccessful attempts, which lead to the conclusion that cell line establishment is controlled by the rare occurrence of a tumor with a built-in potential for long term *in vitro* growth (Freshney ,1986). Yasuda *et al* (2009) pointed out to many factors which must be considered when establishing tumor cell line including: material choice, collection procedures, type of media, source of tumor cells, lapse time between the clinical procedures and preparation for tissue culture and other incidental factors.

In general, cultures derived from embryonic tissue survive and proliferate better than those from the adult. This presumably reflects the lower level of specialization and higher proliferation in embryo. Adult tissue usually have a lower growth fraction and a higher proportion of non replicating specialized cell making the initiation and propagation of cultures more difficult and the life span of the culture often shorter (Davis, 1994; Simmons and Marmion, 1996).

Normal tissue usually gives rise to cultures with finite life span, while cultures from tumors can give rise to continuous cell line (Alley *et al*., 1988). Freshly isolated cultures are known as primary cultures until they are passaged or sub-cultured. The cells are usually heterogeneous and have low growth rate. After several subcultures a cell line will either die out (finite cell line) or become continuous cell line (Nelson Rees *et al*., 1981; Freshney, 1986). The appearance of a continuous cell line is usually marked by an alteration in cytomorphology, an increase in growth rate, a reduction in serum dependence, an increase in cloning efficiency , a reduction in anchorage dependence, an increase in heteroploidy (chromosomal variation) and aneuploidy (divergence from the donor) and increase in tumorigenicity (Freshney, 2001; Ozturk *et al*., 2003).

1.2.3.1 Application of Tumor Cell Line in Pathological and Therapeutical Aspects.

Drug development programs involve pre-clinical screening of vast number of chemicals for specific and non-specific toxicity against many types of cells. The safety evaluation of chemicals involves a range of studies on mutagenicity, carcinogenicity and chronic toxicity (Wilson, 2000). Trivulzio *et al* (1987) mentioned that using *in vitro* assay system for

the screening of potential anticancer agents has been a common practice almost since the beginning of cancer chemotherapy in 1946.

Toxicological agents and cancer chemotherapeutics that are under study share the aim of determining the acute toxicity against variety of cell types. Wilson (2000) added that in both area, the following items must be considered:

• Discovering the potential active compounds as antitumor therapeutics.
• The mode of action by which the compound exert its toxic effect
• Predicting the antitumor activity.
• Determining the toxic concentration range.
• Realizing the relation between concentration and the exposure time.

The experiment of cytotoxic drugs on tumor cell *in vitro* allows the control on physiological and physiochemical environment; give very obvious data and sufficient source for testing drug, together with complete control on drug concentration and the time of exposure more than *in vivo* even with the testing of metabolically labile compounds. The *in vitro* cytotxicity using tumor cell lines usually have simple requirements (using micro-titration system), statistical analyses that are simple and accurate and with low cost (Carney *et al*., 1983; Freshney, 2001).

Freshney (2000) stated that the limits of cytotoxic assay involve; the difficulty to follow up the pharmacokinetics of the compound *in vitro* as *in vivo* and the period of logarithmic phase of cancer cells *in vitro* is shorter than *in vivo*.

1.2.4 Gram positive cell wall composition

1.2.4.1 Peptidoglycan

Peptidoglycan is an essential component of the Gram positive and negative cell wall providing rigid cell structure. Peptidoglycan structure is

composed of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) sugars connected by β-1,4 linkages. A short amino acid chain of 5 residues is added to MurNAc sugars and these peptides are cross-linked by transpeptidases. The cross-linking differs between bacteria and alters the strength of the peptidoglycan (Madigan, Martinko, Parker and Brock, 2003). Peptidoglycan prevents osmotic lysis, maintains bacterial cell shape and is critical in the cell division process (Nanninga.1998). It is the target of many antimicrobials such as the β-lactams (Ghuysen *et al*. 1977).

1.2.4.2 Teichoic acids

Gram-positive bacterial cell walls contain teichoic acids (TA), which are anionic polymers covalently bound to peptidoglycan and called wall teichoic acid (WTC), or anchored to the cytoplasmic membrane, lipoteichoic acid (LTA). Some Gram-positive bacteria do not contain WTC or LTA but contain other anionic polysaccharide. Many roles for teichoic acids have been proposed, including maintenance of cell shape (Steen and Kok, 2003), resistance to antimicrobial peptides (Kovacs and Bruckner, 2006) involvement in biofilm formation (Gross, Cramton and Peschel, 2001) acid tolerance (Boyd and Hamilton, 2000) and the release of secreted proteins (Nouaille and Langella, 2004). The composition and length of both WTA and LTA differ between species and strains (Vollmer and Seligman, 2010).

1.2.5 Genus *Arthrobacter*

The genus *Arthrobacter* was established by Conn and Dimmick (1947) and includes most of the bacteria that exhibit morphological cycle appearance like rod in young cultures and coccus in older cultures (Jones and Keddie, 2006). Although some members of this genus are spheres, occurring in pairs and tetrads, such as *Arthrobacter agilis*.

Jones and Keddie (2006) mentioned that colonies of *Arthrobacter* on nutrient agar medium appear cream or yellow in color, round shape, (3-5) mm in diameter. *Arthrobacter* species have an optimal growth temperature of between 20°C and 30°C and a neutral to slightly alkaline pH (Holt *et al.,* 1994). According to a novel classification system, the *Arthrobacter* genus belongs to the class Actinobacteria, order Actinomycetales, and family Micrococcaceae (Stackebrandt *et al.,* 1997).

The genus Arthrobacter have aerobic metabolism and little or no acid production from glucose, have lysine in the peptidoglycan (Schumann and Kampfer 2009) and a DNA G+C content of 59–66 mol% (Keddie *et al*., 1986). Members of this genus have been isolated from various environments, such as air, soil, fresh water, oil, brine, airborne infections, tobacco leaves, human skin, mural paintings, sewage and activated sludge (Li, 2004; Heyrman, 2005; Margesin, 2004).

It has been reported that some poisonous contaminants and difficult-to-degrade chemical substances such as PCB (Polychlorinated biphenyls), dioxin and oil can be degraded by species of the genus *Arthrobacter* (Fukatsu 2005 and Jussila, 2006).

Arthrobacter is a common genus of soil bacteria (Jones and Keddie, 2006) with all species being generally non-sporulating, Gram-positive and obligate aerobes that exhibit pure respiratory metabolism with the exception of at least two species, *Arthrobacter globiforms* and *Arthrobacter nicotianae* which exhibit anaerobic metabolism (Eschbach and Möbitz, 2003). Due to their metabolic diversity, *Arthrobacter* species have been used in the industrial applications especially in the bioremediation of contaminated water (Eschbach and Möbitz 2003). The usefulness of *Arthrobacter* for bioremediation of industrial wastes rich in nitrates was demonstrated by its ability to remove all nitrate from solutions

containing from 100 to 150 mM NO3− (Piñar and Ramos 1998), while Shen *et al*. (2009) reported on an *Arthrobacter nitroguajacolicus* strain with the capability to transform acrylonitrile into acrylic acid to be use in the manufacture of various plastics and coatings.

Over 70 species of this genus are currently recognized to be occurred in various environments (Euzéby *et al.*, 2011). Members of the genus *Arthrobacter* were described by Conn and Dimmick 1947, and later emended by Koch *et al*. (1995), fall into at least two groups as revealed by the chemotaxonomic studies. The type species, *Arthrobacter globiformis* represents the so-called 'globiformis' group, while the second, the 'nicotianae' group, is centered around the species *Arthrobacter* nicotianae. The globiformis group is characterized by the presence of peptidoglycan of the α3a type (an interpeptide bridge made up of one to four residues of L-amino acids such as L-alanine, L-threonine or L-serine) and of a dehydrogenated menaquinone with nine isoprenoid units. The 'nicotianae' group comprises organisms that have peptidoglycan variation α4a (an interpeptid bridge consisting of a dicarboxylic amino acid such as glutamic acid or aspartic acid) and menaquinones that are completely unsaturated with eight to ten isoprenoid units (Schleifer and Kandler, 1972; Stackebrandt and Schumann, 2006).

1.2.5.1 Membrane structure of *Arthrobacter*

The bacterial cell envelope is critical to maintaining cell shape, withstanding cell turgor pressure, providing protection against harmful agents, mediating cell adhesion and in interactions with host cells (Archibald *et al.*, 1993). Gram-positive cell envelope in compared with Gram negative bacteria (fig. 1.2) consists of only two functional layers that enclose the cellular contents: a cytoplasmic membrane surrounded by a thick cell wall and peptidoglycan, it lacks the outer membrane that present

in Gram negative bacteria (Foster and Popham, 2002). Over 60% of cell wall of Gram-positive bacteria consists of peptidoglycan, according to electron micrographs, and the peptidoglycan of Gram-positive bacteria has a thickness of 30 to 100 nm and may contain up to 40 peptidoglycan layers (Silhavy *et al.*, 2010). Other components such as teichoic acid, which are water soluble polymers of ribitol phosphate or glycerol phosphate, and complex polysaccharides called C-polysaccharides might be also present in the cell wall. This thick peptidoglycan layer lies above the symmetrical cytoplasmic membrane composed of phospholipids. In some Gram positive bacteria peptidoglycan serves as an attachment point for teichoic acids. Teichoic acids can also be attached to the cytoplasmic membrane. Many Gram-positive bacteria also produce capsular polysaccharides linked to the cell wall (O'Riordan and Lee, 2004).

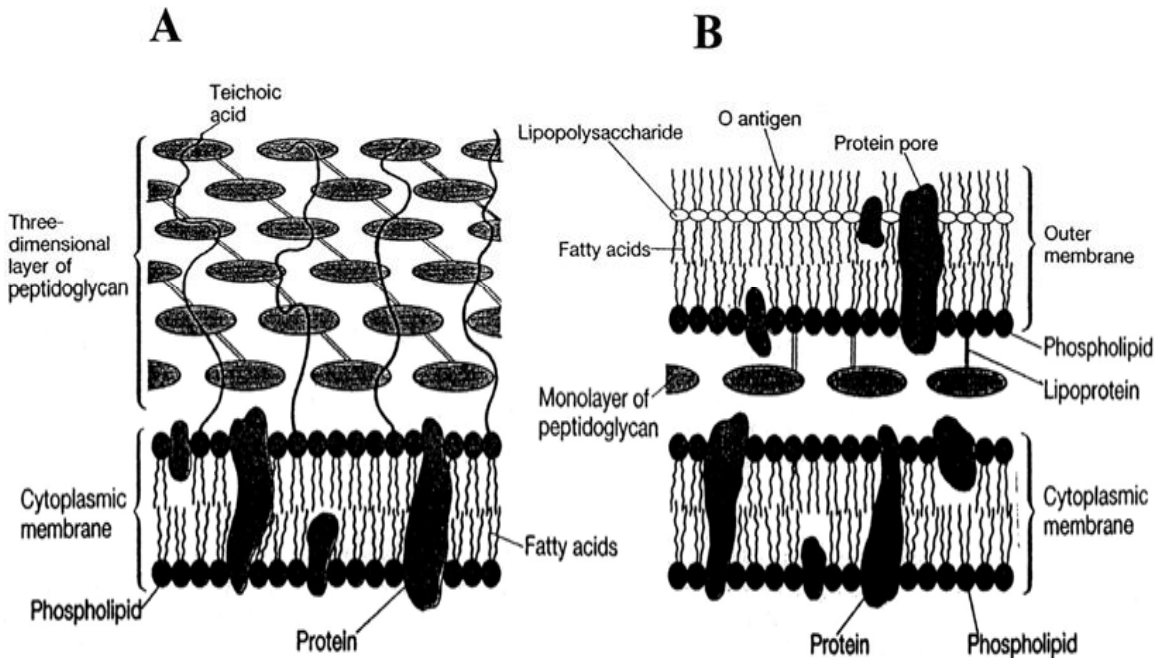

Fig (1.2) Schematic representation the cell envelope of Gram-positive (A) and Gram-negative bacteria (B) from Hamill and Martinac (2001).

1.2.5.2 Cytoplasmic membrane

The selective permeability barrier between the cytoplasm and the cell wall called cytoplasmic membrane, controls substrate flow in and out of the cell (Samuelson *et al*., 2002). Electron microscopic analyses show that the thickness of the membrane is approximately 5-10 nm. The cytoplasmic membrane of bacteria consists of phospholipids and proteins (Dowhan and Bogdanov, 2002). The arrangement of these components in a membrane can be described by the such-called fluid mosaic model. The phospholipids are amphiphilic molecules with polar heads and hydrophobic side chains. The side chains are oriented towards the interior of the membrane and form particular structure called mesosomes or chondroids.

1.2.6 Polysaccharide

Polysaccharides are a large family of biopolymers constituted by sugar monomers linked together by *O*-glycosidic bonds that can be made to any of the hydroxyl groups. Natural polysaccharides can be obtained from different sources, namely algae (alginate), plants (pectin, guar gum), microorganisms (bacteria and fungi such as dextran, xanthan gum and pullulan), and animals (chitin and chondroitin). Instead, semisynthetic polysaccharides have been produced by chemical or enzymatic modification of the parent macromolecules (Honnavally 2003; Holst and Müller-Loennies S 2007).

Depending on source and chemical manipulation, polysaccharides exist in a variety of chemical compositions, architectures, molecular weights and structures. Also polysaccharides can be neutral (pullulan, dextran, cyclodextrins, chitin, starch and cellulose), positively charged (chitosan), or negatively charged (alginate, heparin, hyaluronic acid and pectin) polyelectrolytes. The glycosylic bonds involved in the monomer

condensation determine the conformational structure of these macromolecules which can be linear, branched, or circular. The composition and architecture of polysaccharides dictates their physicochemical properties including solubility, gelation, and surface properties (Chandra and Rustgi 1998; Meyers *et al.,* 2008)

Typically, polysaccharides can interact with living cells displaying biological properties such as antioxidant, antimicrobic, cell differentiation, anticoagulant, immunostimulant (Sun *et al.,* 2009). Heparins and fucose-containing sulfated polysaccharides, for example, possess blood-anticoagulant activities (Smit *et al.,* 2004). Several polysaccharides and glycoproteins from seaweed have immunostimulant, antitumoral, or antiviral activity. β-glucans located in microorganisms and cereals stimulate the immune system, modulating humoral, and cellular immunity, and thereby have beneficial effect in fighting infections (bacterial, viral, fungal, and parasitic) (Mantovani and Bellini 2008). Hyaluronans are involved in a number of cell functions in mammalians and have a role in inflammation and cancer biogenesis.

The use of polysaccharides as soluble drug carriers in bioconjugation technology represents one of the most challenging application of these materials (Golenser *et al.,* 2004; Payne *et al.,* 2007). The hydroxyl, carboxyl, and amino groups naturally present or artificially introduced in the polymer backbone can, in fact, be exploited for direct or spacer mediated drug conjugation yielding macromolecules bearing high number of drug units. The conversion of drugs into macromolecular prodrugs can improve their poor physicochemical and biopharmaceutical properties thus enhancing their therapeutic value. Anticancer drugs, for example, are usually low soluble molecules which can easily undergo chemical or enzymatic inactivation. Conjugation to polysaccharides can increase their solubility and protect them from inactivation thus endowing

derivatives with improved therapeutic performance. Additionally, the macromolecularization alters dramatically the pharmacokinetic properties of drugs prolonging their circulation time in blood and promoting the passive accumulation into permeable tissues, namely liver or disease tissues. In particular, macromolecules have been demonstrated to accumulate passively in solid tumors by enhanced permeability and retention (EPR) as a consequence of the leaky vasculature of these tissues and slow lymph drainage (Kim and Park, 2007). Additionally, polysaccharides can inherently recognize specific receptors located on tumor cells thus conveying targeting properties. Tumorotropic properties can be also conveyed by altering the chemical structure, namely polymer size, charge, and by introducing targeting moieties (Sugahara and Okuno, 2001; Rinaudo *et al.,* 2006) Therefore, the combination of structural and biological properties of polysaccharides, together with the possibility of chemical modification by simple procedures, makes these materials suitable candidates for development of soluble conjugates for anticancer drug delivery.

1.2.6.1 Polysaccharide and apoptosis

Chemotherapy is one of the major therapeutic modalities for cancer. Conventional chemotherapeutic agents are highly toxic to normal tissues and are not successful for complete remission of tumors and to prevent metastasis. During the past three decades, many polysaccharides and polysaccharide protein complexes have been isolated from mushrooms, fungi, yeasts, algae, lichens, plants, and bacteria with medicinal properties. The biological activity of the polysaccharides has attracted more attention recently in the biochemical and medical areas because of their immune modulatory and antitumor effects. The ability of bioactive polysaccharides and polysaccharide bound proteins to modulate so many

important immune functions may be due to the structural diversity and variability of these macromolecules. A successful approach in cancer therapy is to trigger apoptosis but it is often complicated by development of multi drug resistance (MDR) mechanisms.

Apoptosis is a physiological process of cell elimination, and DNA fragmentation is one of the hallmarks of cell apoptosis, apoptosis can be defined as a carefully regulated process, characterized by specific morphologic and biochemical features. It is initiated by both physiologic and pathologic stimuli, and its full expression requires a signaling cascade in which caspase activation plays a central role (Zeiss, 2003). Apoptosis is a genetically controlled process that plays important roles in embryogenesis, metamorphosis, cellular homeostasis, and as a defensive mechanism to remove infected, damaged or mutated cells. Although a number of stimuli trigger apoptosis (Shankar and Srivastava, 2007).

Apoptotic cells can be recognized by stereotypical morphological changes: the cell shrinks, shows deformation and loses contact to its neighboring cells. Its chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding, and finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin, and organelles. The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response (Saraste, 2000; Nicotera and Melino, 2004). Apoptosis may be essential for the prevention of tumor formation, and its deregulation is widely believed to be involved in pathogenesis of many diseases, including cancer (Evan and Vousden, 2001).

Polysaccharides are able to down regulate P-glyco protein, and reverse MDR (Wei et al. 2008). Polysaccharides from natural resources

exert their tumor inhibition effects are reviewed by Zong *et al* (2012) and can be assigned into the following four aspects: (1) the prevention of tumor genesis by oral consumption of active preparations (2) direct anticancer (3) immune potentiation activity in combination with chemotherapy and (4) the inhibition of tumor metastasis.

Cellular proliferation depends on the rates of cell division and death and, thus, many anticancer drugs have been used to prevent cancer cell division in order to inhibit cancer cell proliferation. Control of the cell cycle progression in cancer cells was considered to be a potentially effective strategy for the control of tumor growth as molecular analyses of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies (Mantena SK *et al*., 2006), number of cells in the G0/G1 phase was significantly augmented by treatment of polysaccharide therefore, the cell cycle arrest in the G0/G1 phase might be one mechanism of the anticancer effects of polysaccharide.

Apoptotic defects are required to complement proto oncogene activation, as many oncoproteins will produce that increase cell proliferation and the life span of the cells (Horak *et al*., 2008).

Chapter Two
Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Biological and Chemical Materials

Material	Company (origin)
Peptone, Tryptone, Soluble Starch, Agar	Himedia (India)
Urea powder, Yeast extract, L-glutamine.	Oxiod (England)
Glucose, Ethanol Absolute, Ethylene Diamine Tetra Acetic Acid (EDTA), Safranine, Giemsa stain, Glycerol, Bovine serum albumin, Malachite Green.	BDH (England)
NaCl, $NaHCO_3$, Iodine, Phenol Red.	Merck (Germany)
Agar, Tris-HCl, KCl.	Fluka (Switzerland)
Methyl Red, H_2SO_4.	Riedel DeHaeny (Germany)
Crystal violet, Sepharose-Cl-6B.	Pharmacia (Sweden)
K_2HPO_4, KH_2PO_4, Formalin.	Analar (England)
Coomassie Brilliant Blue G-250.	LKB (Sweden)
Phenol, Trypsin, Fetal Calf Serum.	Sigma (USA)

Colchicine.	Unipharma (Syria)
Chloroform	Applichem (Germany)
Agarose, Sodium dodecyl sulfate (SDS), Potassium acetate.	Promega (USA)

2.1.2 Enzymes (Sigma, USA)

DNase**,** RNase**,** Proteinase K and Steptoccocal MNase

2.1.3 Equipment and apparatus

Equipment and apparatus	**Company (origin)**
Autoclave	Express (Germany)
Bench centrifuge	Netheler and Hinz
Compound light microscope	Olympus (Japan)
Disposable micropipette tips	Walter (Germany)
Disposable Petri-dishes	Grenier (Germany)
Distillator	Kottermann (Germany)
Incubator	Memmert (Germany)
Lyophilizer	Fisher (U.K)
Micropipette	Gilson (France)
Millipore filters unit	Gallen Kamp (England)
Pasteur pipette	Gilson (France)

pH- meter	Metter-Tolledo (U.K)
Sensitive balance	Ohaus (Germany)
Vortex	Giffin (England)
Water bath	Gallen Kamp (England)
UV-Visibile Spectrophotometer	Shemadzu (Japan)
CO2 Incubator	Gallenkamp (U.K)
Inverted microscope	Gallenkamp (U.K)
Shaking Incubator	GLF (Germany)

2.1.4 Culture Media

2.1.4.1 Ready to Use Media

Media	**Company (Origin)**
MR-VP Broth	Himedia (India)
Urea Agar Base	Himedia (India)
RPMI-1640 with HEPES	Sigma (USA)
Simmon's Citrate Agar	Difco (U.S.A)
Nutrient Agar	Fluka (Germany)

These media were prepared and sterilized as recommended by the manufacturing companies. All mentioned media were sterilized by autoclaving, except the RPMI-1640 has been sterilized by filtration.

2.1.4.2 Laboratory Prepared Media

A. Peptone water for Indol test (Collee *et al*., 1996).

It was prepared by dissolving 20 g of peptone and 5 g of NaCl in 1000 ml of distilled water and divided in test tubes each tube containing 5 ml then sterilized by autoclaving. It was used for Indol test.

B. Urea agar medium (Collee *et al*., 1996).

It was prepared by adding 24 g of urea agar base to 950 ml of distilled water, pH was adjusted to (6.8 - 7) and sterilized by autoclaving, then left to cool to 50 °C, A portion of 50 ml of 20% urea solution which sterilized by filtration using (0.22 micrometer) filter unit was added. After that, 5 ml were dispensed in sterile test tube and left declined to solidify as slants.

C. Luria-Bertani (LB) Broth (Maniatis *et al*., 1982)

Ingredients	Concentration (g)
Tryptone	10
Yeast Extract	5
NaCl	5

PH was adjusted to 7.2 and sterilized by autoclaving. For LB agar preparation 1.5% agar was added.

D. Starch agar (Corry *et al*., 2003)

Ingredients	Concentration (g/L)
Soluble Starch	10
Yeast Extract	3
Agar	12

All ingredients were dissolved in 1L distilled water, the pH was adjusted to 7.2 before sterilized by autoclaving.

2.1.5 Cell line (Chiang *et al.*, 1998)

HepG2 (Hepatocellular carcinoma, human) Cell line passag 328 was kindly provided by the Biotechnology Research Center, at Al-Nahrain University.

2.1.6 Reagents and Dyes for *Arthrobacter* Identification

A. Kovac's Reagent (Collee *et al.*, 1996)

This reagent was prepared by dissolving 5 g of ρ-Dimethyl aminobenzaldehyde in 75 ml of isoamyl alcohol, then 25 ml of concentrated HCl was added.

B. Voges-Proskaur VP Reagent (Atlas *et al.*, 1995)

VP1: 40% of potassium hydroxide in distilled water.

VP2: 5 g of α-naphthol in 100 ml of absolute ethanol.

C. Methyl Red Reagent (Collee *et al.*, 1996)

This reagent was prepared by mixing the following components

Ingredients	Concentration (g/L)
Methyl Red	0.05 g
Ethanol	150 ml
Distilled Water	100 ml

E. Gram Staining pigment and solutions were prepared according to Atlas *et al.*, (1995).

2.1.7 Buffers and Solutions of polysaccharide Extraction

A. Phosphate Buffer (0.025M) (Silipo *et al.*, 2002)

It was prepared by dissolving 1.21 g K_2HPO_4 and 0.34 g KH_2PO_4 in 1L distilled water**,** the pH was adjusted to 7.2 and sterilized by autoclaving before storing at 4°C.

B. Ethylene-diamine-tetra-acetic acid (EDTA) Solution /0.5M (Chandan and Fraser, 1994)

It was prepared by dissolving 8.1 g EDTA in 50 ml phosphate buffer (0.025M).

C. DNase Solution (Maniatis *et al*., 1982)

A quantity of 1 mg of DNase was dissolved in 10 ml of 10 mM Tris-base (pH 7.5) and store at -20ºC.

D. RNase Solution (Maniatis *et al*., 1982)

RNase powder (in concentration of 1mg/ml) was dissolved in 10mM Tris-base (pH 7.5) and 15mM NaCl, then heat at 100ºC for 15 min and allowed to cool slowly to the room temperature before Store at -20ºC.

E. Proteinase K Solution (Maniatis *et al*., 1982)

A quantity of 1 mg was dissolved in 10 ml distilled water and stored at -20ºC.

2.1.8 Solutions for Determination of Protein Concentration (Bradford, 1976)

A. Coomassie Brilliant Blue G-250 Stain.

It was prepared by dissolving 0.1 g of Coomassie brilliant blue-G-250 in 50 ml of 95% ethanol, then 100 ml of 85% phosphoric acid was added with agitation, the volume was completed to 1L with distilled water, then filtrated via Wattman filter paper (No.1) and kept in a dark bottle.

B. Tris-HCl Buffer.

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

C. Bovine Serum Albumin (BSA).

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

2.1.9 Solutions of Carbohydrate Determination Concentration (Dubois *et al*., 1956)

A. Glucose Stock Solution.

It was prepared by dissolving 1 mg of glucose in 10ml distilled water.

B. Phenol Solution (5%) Dissolved in Distilled Water.

C. H_2SO_4 Solution (98%).

2.1.10 Solutions of Partial Purification of Polysaccharide (Silipo *et al*., 2002)

- Phosphate buffer (0.025M) as described previously in item (2.1.6. A).

2.1.11 Media and Solutions used for the Animal cell culture (Freshney *et al*., 2000).

A. Benzyl penicillin stock solution Contents of one vial (106 IU) was dissolved in 5 ml of sterile distilled water which is consider as stock solution with concentration was [200000 IU/ml] and stored at-18°C.

B. Colchicine stock solution

Twenty gram were dissolved in 20 ml distilled water to reach a concentration of 1 g/ml. The stock was sterilized by filtration using (0.22 micrometer) filter unit.

C. Sodium Bicarbonate Solution

The solution was prepared by dissolving 4.4g of NaHCO3 in 100ml of sterile distilled water. This solution was kept at 4°C until use.

D. Phosphate Buffer Saline

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

E. Trypsin solution

It was prepared by dissolving 1 g of trypsin powder in 100 ml of phosphate buffer saline and sterilized by filtration, then dispensed into 20 ml aliquots and stored at -20 °C.

F. Versene solution

Versene solution was prepared by dissolving 1 g of ethylene-diamine-tetra acetic acid (EDTA) in 100 ml of phosphate buffer saline, then the solution was dispensed in 20ml aliquots and sterilized by autoclaving before storing at 4°C

G. Trypsin – Versene Solution

It was prepared by mixing 20ml of trypsin solution, 10ml of versene solution and 370 ml phosphate buffer saline. The mixture was stored at 4°C.

H. Rosswell Park Memorial Institute (RPMI) 1640 Complete Medium

Ready to use RPMI-1640 medium with HEPES and L-glutamine was used and supplemented with the following ingredients as described by Freshney, (2010):

Ingredients	Concentration (g/L)
Penicillin G	104 IU
Sodium bicarbonate	1%
Fetal calf serum	10%

2.1.12 Buffer and Solutions of cell digestion

A. MNase buffer (O'Neill and Turner, 2003).

It was prepared by dissolving the following ingredients in 500 ml of distilled water.

Ingredients	Concentration (g/L)
$CaCl_2$	4 mM
KCl	25 mM
$MgCl_2$	4 mM
Glycerol	125 ml
Tris-Hcl	50 M

The volume was completed to 1L with distilled water.

B. SDS solution (O'Neill and Turner, 2003)

It was prepared by dissolving 10 gm of SDS in 90ml of sterile distilled water then the volume was completed to 100 ml with sterile distilled water.

C. EDTA (0.5M) solution (O'Neill and Turner, 2003)

It was prepared by dissolving 1.45gm of EDTA in 10ml of sterile distilled water.

D. Proteinase K Solution

A quantity of 1mg was dissolved in 10ml sterilized distilled water and stored at -20ºC.

2.1.13 Buffers and Solutions for DNA extraction

A. Chloroform

B. Chloroform phenol solution (Sambrook and Russell, 2001)

It was prepared by mixing 50 ml of chloroform with 50 ml of phenol solution.

C. Potassium acetate (Sambrook and Russell, 2001).

It was prepared by dissolving 10 g of potassium acetate in 100 ml distilled water.

D. Ethanol 98%

E. TE buffer (Maniatis *et al*., 1982)

It was prepared by dissolving 0.12 gm Tris-HCl and 0.029 g of EDTA in 100 ml sterile distilled water.

2.1.14 Buffers and Solutions of Electrophoresis

A. TAE buffer

It was prepared by dissolving 400 mM Tris-acetate and 10 mM EDTA in 1L distilled water, PH was adjusted to 8.2.

B. Agarose

Agarose powder 1.2 g was dissolved in 100 ml of 1X TAE buffer and the slurry was heated using microwave for 2min until dissolved.

C. Ethidium bromide (Sambrook and Russell, 2001).

It was prepared by dissolving 10 mg of ethidium bromide in 1 ml distilled water, then stored at 4°C.

D. Gel loading buffer (Sambrook and Russell, 2001)

It was prepared by mixing Bromophenol blue (0.25% w/v) and sucrose (40% w/v) in sterile distilled water.

2.2 Methods

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

A. Moist heat sterilization

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

B. Dry heat sterilization

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

C. Filtration (membrane sterilization)

Solutions that sensitive to heat were sterilized by filtration using millipore filters 0.22µm diameter.

2.2.2 Samples Collection

Moisturized soil samples were collected from ten different sites of three region in Baghdad (Jadriah, Karradah and Bayaa). Samples were taken from both surface and 30 cm deep from surface. Samples were transferred to the laboratory in sterile plastic bags for analysis.

2.2.3 Isolation of *Arthrobacter*

Five gram of each of the soil sample was suspended and well mixed in a volumetric flask containing 45 ml of sterile tap water, to obtain 10^{-1} dilution. Serial dilution (10^{-2}, 10^{-3}, 10^{-4}, and $10^{-5)}$ were

prepared. Then 0.1 ml of each dilution was plated (by spreading with the aid of L-shape glass rod) on surface of solidified nutrient agar.

Plates were incubated (inversely) at 30°C for 48 hr. After incubation, single colony were picked with a sterile microbiological loop and streaked on fresh nutrient agar plates to obtain pure culture of isolate. The suspected bacterial isolate was subjected to cultural microscopical and morphological test.

2.2.4. Identification of bacteria

Suspected bacterial isolates were identified by cultural, microscopic and biochemical tests.

2.2.4.1 Cultural Characteristics

Shape, color, size, edge, odor and viscosity of the colonies grown on nutrient agar plate.

2.2.4.2 Morphological Characteristics (Atlas *et al.*, 1995).

A single colony was transferred by a loop onto clean slide. The smear was stained with crystal violet, treated with iodine, decolorized with absolute alcohol and counterstained with safranine, then examined under the oil immersion lens of the compound light microscope to detect gram reaction, shape and growing of cell of isolated bacteria.

2.2.4.3. Biochemical Tests

The following biochemical tests for identification of *Arthrobacte*r were performed:

A. Methyl-red and Vogas Proskauer (Maza *et al*., 1997)

Test tube containing MR-VP broth were inoculated with fresh culture of each bacterial isolate and incubated at 37°C for 24 hr. For methyl red test about five drop of methyl red reagent (2.1.5) were added and positive result was detected by bright red color. While the Vogas-Proskauer test was performed by adding 1ml

VP1and 3ml VP2 reagents (2.1.6) to 5 ml of the bacterial culture (with shaking) for 30 seconds. Formation of pink color indicates positive result.

B. Starch hydrolysis (Harely and Prescott, 1996)

Starch agar medium was inoculated with single streak of bacteria and incubated at 37°C for 48 hr. After incubation the plates were flooded with iodine solution. Development of yellow color around the colonies indicates the hydrolysis of starch.

C. Urease production (Atlas *et al*., 1995)

Christensen urea agar slants were inoculated with fresh culture of each suspected isolates and incubated at 37°C for 24 – 48 hrs. Appearance of pink color on slant indicates a positive reaction.

D. Catalase production (Maza *et al*., 1997)

This test was performed by adding drops of hydrogen peroxide (3%) on a single bacterial colony grown on trypticase soya agar. Production of gaseous bubbles indicates ability of the isolate to produce catalase enzyme.

E. Oxidase production (Maza *et al*., 1997)

Filter paper was saturated with oxidase reagent (2.1.6), and then one colony of the suspected isolate was rubbed on the filter paper with a sterile wooden applicator stick. An immediate color change to deep blue indicates ability of isolate to produce oxidase enzyme.

F. Indole Production (Collee *et al*., 1996)

Test tubes containing peptone water broth were inoculated individually with fresh culture of each suspected isolate and incubated at 37°C for 24-48 hours. After that 0.5 ml of Kovac's

reagent was added. Presence of red ring on surface of the medium indicates a positive result.

G. Endospore stain (Dustman *et al.*, 2003).

Smear of freshly culture was heat fixed on microscopic slide after that put the slide over beaker containing boiling water under bunsen burner then cover the slide with Malachite Green for 3 - 5 minutes, allow the slide to cool and rinse it with tap water to remove excess stain then cover the smear with Safranin for two minutes, rinse the slide with water to remove excess stain and left the slide to dry view under a microscope, the vegetative cells will appear red and the spores will appear green.

H. Citrate utilization (Collee *et al.*, 1996).

Simmon's citrate agar slant was inoculated with isolated bacterial culture by streaking with sterile loop and incubated at 37°C for 24-48 hour. A positive result was indicated by changing the colour of medium from green to blue.

2.2.5 Maintenance of bacterial isolates

Maintenance of bacterial isolates was performed according to Sambrook and Russell (2001) as follows:

A. Short –term storage

Colonies of bacteria were maintained for periods of few weeks on the surface of nutrient agar media; the plates were tightly wrapped in parafilm and stored at 4°C.

B. Medium –term storage

Bacterial isolates were maintained in the stab culture for a long periods of a few months. Such as cultures were prepared in small screw capped bottles containing 2-3 ml of agar medium, the cultures were inoculated using a sterile straight platinum wire that

is dipped into dense liquid culture of bacteria and then stabbed deep into agar medium. After proper incubation, the cap was wrapped tightly with the parafilm to prevent desiccation of the medium and kept at 4 °C.

2.2.6 Extraction of *Arthrobacter* Polysaccharide

2.2.6.1 Cells preparation (Silipo *et al.*, 2002).

Bacterial cells were activated by growing in a flask containing 25 ml of LB broth before incubation at 30 ºC for 18 hrs. The fresh culture was used to inoculate 3-3.5 L of LB broth suspended in a 500 ml conical flask filled with 200 ml broth. The inoculated flasks were incubated at 30 ºC for 24 hr with shaking at 150 rpm. Cultures were centrifuged (3000 rpm, 15 min), and pellets were washed twice with phosphate buffer. Cells were suspended in phosphate buffer containing 0.5% formalin (pH= 7.2) before keeping at 4ºC for18 hrs. After that, the cells were centrifuged (3000 rpm, 15min) and washed with phosphate buffer. Finally, cells were dried to one-tenth of the original sample size by using cold acetone.

2.2.6.2 Polysaccharides Extraction (Chandan and Fraser, 1994)

- Twenty gram of the dried cells were suspended in 50 ml phosphate buffer; 0.5 ml of 0.5M EDTA solution was added. The suspension was then homogenized with magnetic Stirrers for 2 min.
- The mixture was autoclaved at 121ºC for 10 min and left to cool at room temperature
- DNase and RNase solutions were added to the mixture in final concentration of 1µg/ml for each solution and incubated at 37ºC for 10 min.

- Proteinase K solution in a final concentration of 1µg/ml was added to the mixture and incubate at 56ºC for 20 min and then the mixture was left to cool at room temperature
- The extracted mixture was centrifuged at 10000 rpm for 15 min. Two phases were formed; the aqueous phase (upper phase) was aspirated off with a sterilized Pasteur's pipette and dialyzed for 4-6 days against distilled water at 4ºC with changing the water every day.
- The dialyzed sample that contained polysaccharide was lyophilized to obtain crude polysaccharide.

2.2.7. Partial Purification of Polysaccharide

This was performed by Gel Filtration Chromatography according to the method described by Johnson and Perry, (1976) as fallow:

2.2.7.1 Preparation of Sepharose Cl-6B Column

According to the manufacturer instruction the gel was washed several times with 0.025 M phosphate buffer to keep the pH at 7.2 (2.1.7.A). Gas bubbles were degassed using a vacuum pump. Then the gel was slowly poured into the inner side of the column (3 x 50 cm) and equilibrated with the phosphate buffer with a flow rate 30 ml/hour.

2.2.7.2 Purification Procedure

Crude polysaccharide (30 mg/ 5 ml) was added gently through the sepharose Cl-6B column that has been previously equilibrated with the phosphate buffer using a pasture pipette. Fractions have been collected at a flow rate of 30 ml/hr and with a fraction size of 3 ml/tube. Fractions were collected and examined as follows:

- Absorbance at 280 nm was determined for detecting the contaminated proteins within fractions (Buruck *et al*., 1982).
- Carbohydrate concentration was estimated according to method described by Dubois *et al*., (1956).

- Absorbance at 260 nm was determined for detecting the nucleic acids (Swada *et al*., 1984).

2.2.8 Chemical Analysis of Polysaccharide

2.2.8.1 Determination of Protein Concentration

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

- A standard curve of bovine serum albumin was plotted by using different concentrations of BSA stock solution (as prepared in item 2.1.8 C), then volumes were prepared as below

BSA (µl)	Tris-HCl Buffer (µl)	Protein Amount (µg)	Final Volume (ml)
20	80	20	0.1
40	60	40	0.1
60	40	60	0.1
80	20	80	0.1
100	0	100	0.1

- A volume of 2.5 ml of Coomassie brilliant blue G-250 dye was added, mixed and left to stand for 2 min at room temperature.
- The absorbance at 595 nm was measured; the blank was prepared from 0.1 ml of Tris-HCl buffer and 2.5 ml of the dye reagent.
- A standard curve was plotted between the BSA concentrations against corresponding absorbance of the bovine serum albumin.
- Protein concentration of the polysaccharide sample was estimated by subjected 0.1 ml of 1mg/ml polysaccharide solution (dissolved in Tris-HCl buffer), to the same previous addition and the absorbance at

595 nm was read. Protein concentration was calculated from the standard curve.

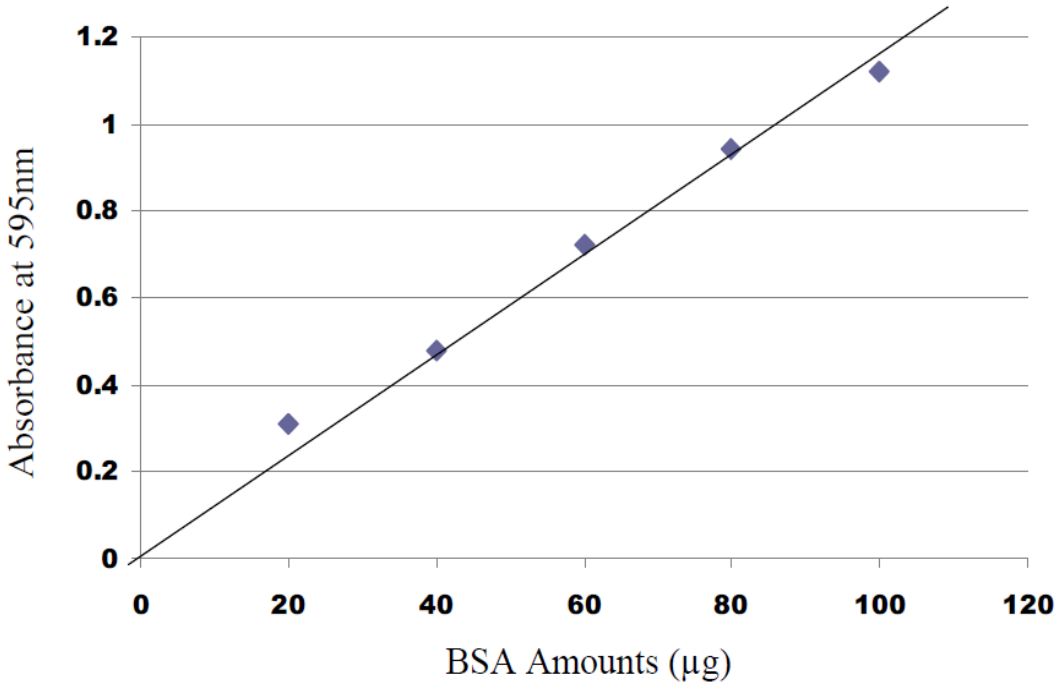

Fig. (2-1): Standard Curve of Bovine Serum Albumin Described by (Method of Bradford, 1976).

2.2.8.2 Determination of Carbohydrate Concentration

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

- Different concentrations (10, 20, 40, 60, 80, 100 µg/ml) were prepared from glucose stock solution (item 2.1.9) with final volume of 1 ml. Then 1 ml of phenol solution (5%) was added to each tube with mixing.
- A volume of 5 ml from H_2SO_4 was added to the mixture with vigorous mixing, then left to cool to the room temperature.
- Absorbance at 490 nm was measured; the blank was prepared from 1 ml of distilled water, 1 ml phenol solution (5%) and 5 ml of H_2SO_4.

- ❖ The standard curve was plotted between glucose amounts against corresponding absorbance of the glucose concentrations.
- ❖ Carbohydrate concentration of polysaccharide was estimated by subjected 1 ml of 1mg/ml polysaccharide solution (dissolved in distilled water) to the same previous addition and absorbance was read at 490 nm. Carbohydrate concentration was calculated from the standard curve.

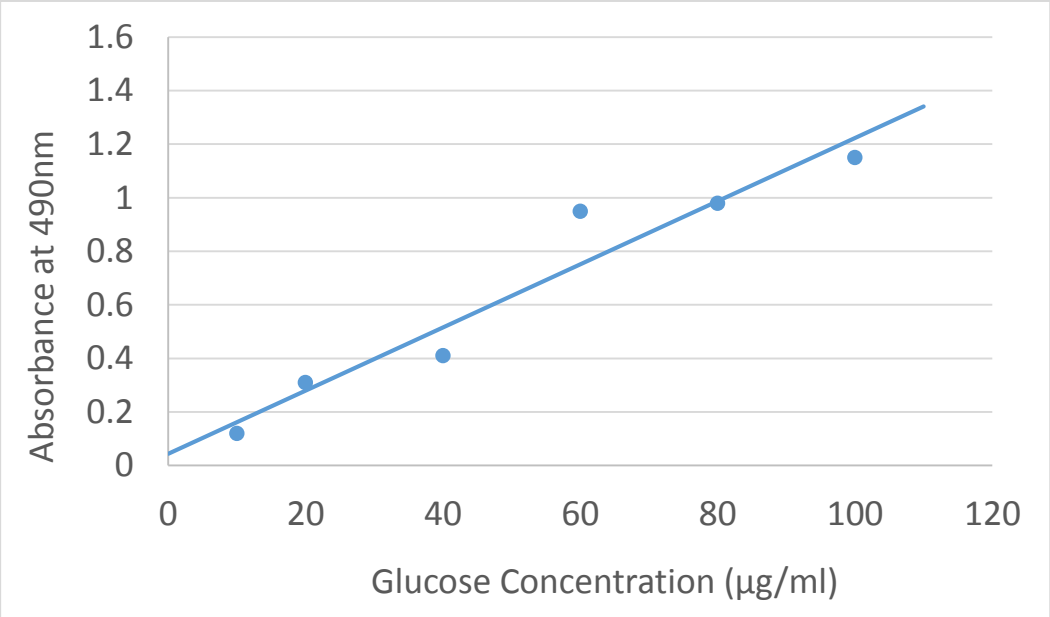

Fig. (2-2): Standard Curve of Glucose Concentration Described by Dubois *et al*., (1956).

2.2.9 Effect of Partially Purified *Arthrobacter* Polysaccharide on DNA of HepG2 tumor cell line.

This test was performed as follow:

2.2.9.1 Maintenancing cell lines (Freshney, 2000)

After cells in the flask were formed confluent monolayer, the following protocol was performed.

a) Growth medium was decanted off and the cell sheet washed twice with phosphate buffer saline.

b) Two to three mls of trypsin/versine solution were added on side of the flask opposite to the cells. The flask was turned over to cover the monolayer completely with gentle rocking before incubation at 37°C for few minutes until the cells detached from the flask.

c) Medium (20 ml of RPMI-1640) was added, and cells were dispersed by pipetting in growth medium over the surface bearing the monolayer.

d) After the medium was added, cells were redistributed at the required concentration into culture flasks and incubated at 37°C with 5% CO_2.

2.2.9.2 Treatment of HepG2 tumor cell with polysaccharide and colchicine

HepG2 tumor cell were cultured in 125 ml flasks containing 20 ml of complete RPMI-1640 medium for 48 hr at 37°C under 5% CO2. After the formation of the confluent monolayer, flasks were treated as follows:-

a) Five flasks were treated with colchicine at a final concentration of 50 µg/ml, then flasks were incubated at 37°C for 24 hr under 5% CO_2.

b) Five flasks were treated with the partially purified *Arthrobacter* polysaccharide at a final concentration of 1 mg/ml. Flasks were incubated at same above condition.

c) Five flasks were used as a control (untreated) and incubated for further 24 hrs at 37 °C for 24 hrs with 5% CO_2.

After incubation, the treated and untreated plates were mitotically shack-off by hard tapping the plates on the bench, in order to deattachment cells from the plates and suspended in the medium. To ensure the complete cell deattachment, 2-3 ml trypsin were applied for few minutes. Cells (both treated and untreated) were fixed by 10%

formaldehyde for 10 min, then the fixation was stopped by adding 5% glycine (2.5 M).

Cells were spined in portable centrifuge (1500 rpm) for 10 min, supernatant was discard and the pellets were suspended in 3ml of MNase cleavage buffer, and cells concentration was adjusted to 4×10^6 cell/ml using the Haemocytometer. Cells, then, were flash freezed and kept at -20 °C until use (Spetman *et al.*, 2011).

2.2.9.3 MNase cleavage and cell digestion (Druliner *et al.*, 2013)

a) One hundred µl cells from each colchicine treated cells, polysaccharide treated cells and untreated cells were used.

b) MNase titration reaction of 0, 5, 7.5 and 10 u/ml MNase in MNase cleavage buffer was performed for 4 min at 37°C.

c) Reaction was stopped by adding 20µl of 50 mM EDTA, followed by adding 100µl MNase cleavage buffer for each tube.

d) MNase - digested cells were then treated with proteinase K at a final concentration of 0.2 mg/ml and SDS at a final concentration of 1%.

e) Tubes were overnight incubated at 65°C.

2.2.9.4 DNA extraction

DNA was extracted depending on Sambrook and Russell, (2001) protocol with minor modifications follow:

a) An equal volume of chloroform-phenol (1:1) was added to each tube sample, vortexed for 30 seconds until emulsion forms.

b) The mixture was Centrifugated at 14000 rpm for 5min.

c) The organic phase was removed from the bottom of tube and the aqueous phase was resuspended with an equal volume of chloroform.

d) Mixture was vortexed for 30 seconds and centrifuged for 5 min at 16000 rpm.

e) Aqueous phase was aspirated and transferred into a new tube.

f) Ten percent of 3M of potassium acetate was added, vortexed for seconds then twice sample volume of the cold absolute ethanol was added.

g) Mixture was kept in freeze for 15-20 min.

h) Mixture was centrifuged at 13000 rpm for 20 min at 4°C.

i) Ethanol was discarded and tubes were washed with 70% ethanol.

j) The 70% ethanol was aspirated and tubes were left to dry.

k) DNA samples were suspended with 50 ml of TE buffer.

2.2.9.5 Agarose gel electrophoresis (Sambrook and Russell, 2001).

Electrophoresis was carried out on a horizontal submarine electrophoresis apparatus. Agarose powder (1.2%) was dissolved in 1X TAE buffer and the slurry was heated using microwave for 2min until dissolved. The solution was cooled to 50°C and ethidium bromide was added to a final concentration of 0.5 µg/m. Electrophoresis was performed at 100 volts for 45min. The DNA bands were visualized on a short wave of UV transilluminator and photographed by (Gel Doc XR+, Bio-Rad, Germany) imaging system.

Chapter Three
Results and Discussion

3. Results and Discussion

3.1 Isolation of *Arthrobacter*

When soil samples were collected from ten locations in Baghdad city, suspected isolates of *Arthrobacter* were detected in only one location. The suspected *Arthrobacter* spp were isolated by culturing 0.1 ml of the one-tenth diluent of soil sample on surface of nutrient agar in a petri-dish before incubating at 30ºC for 48 hrs. In fact, soil locations of sampling were characterized by being wet, partly shaded and covered or mixed with dead plant leaves and stems. After incubation, the isolates were subjected to the identification.

Arthrobacter spp may account up to 60% of the total bacterial occurrence in the soil, but its presence decreases with increasing soil acidity and soil dryness (Holt *et al.,* 1994; Guadalupe and Ramos, 1998). Other studies indicated the possibility of isolating *Arthrobacter* sp. from the co- buried wet soil of aerial plant material (Zhao *et al.,* 2009 and Wang *et al.,* 2010).

3.2 Identification of bacterial isolates

3.2.1 Culture characteristics:

Culture Characteristics:

Growth of the suspected bacterial isolates on nutrient agar gave creamy or yellow colonies round in shape, (3-5) mm in diameter. In this regard, Holt (1994) mentioned that such characteristics are similar to those of *Arthrobacter*.

3.2.2 Microscopic Characterization

The suspected isolates were first identified depending on their Gram stain and other microscopic characteristics. Results showed that the isolates were found to be Gram positive, rod-cocci and non-spore formers, such properties are coincided with those of *Arthrobacter* (Jones and Keddie, 2006).

Those suspected isolates were further undergone morphological and microscopic examinations depending on their life cycle. Because of the unique life cycle *Arthrobacter* have, they will appear slender rods when grown in young and rich nutrients cultures. But after 72 hrs.from being cultured and when nutrients be depleted, the cells will became very short rods or coccids (Eschbach *et al*., 2003).

Depending on the above results, two isolates (A1 and B1) exhibited a pattern of life cycle similar to that of *Arthrobacter*, therefor, they were subjected for final identification by the biochemical tests.

3.2.3 Biochemical Characterization:

When nine of the selected biochemical tests were performed on the two selected isolates results declared that only one isolate (namely B1) gave negative reactions for each of citrate utilization, urease, and methyl red tests, but it was positive for each of indole, Voges-Proskaur, catalase, and oxidase tests as shown in table (3-1). Such biochemical characterization comes in accordance with that of Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1994).

Table (3-1): Biochemical properties of *Arthrobacter* isolate A1 and B1

Biochemical test	Isolate A1	Isolate B1
Oxidase	Negative	Positive
Catalase	Positive	Positive
Indole	Negative	Positive
Voges-Proskaur	Positive	Positive
Urease	Negative	Negative
Citrate Utilization	Negative	Negative
Methylen Red	Positive	Negative
Spore Forming	Positive	Negative
Starch hydrolysis	Positive	Positive

3.3 Polysaccharide from *Arthrobacter* Isolate B1:

Polysaccharide is the major constituent of the outer membrane of Gram-positive bacteria. Due to the importance of bacterial polysaccharide as a membrane barrier, bacterial recognizing site, induction of immune system and therapeutic activity (Tanamoto *et al*., 2001), various procedures have been developed for extraction of bacterial polysaccharide depending on the chemical nature, bacterial type and purpose of polysaccharide (Apicella, 2008).

The polysaccharide of *Arthrobacter* B1 isolates was extracted using a procedure developed by Chandan and Fraser (1994) which depends on a combination between the application of pressure and hot water for bacterial cell destruction, and hydrolytic enzyme treatment for protein and nucleic acid removal.

When isolate B1 of *Arthrobacter* was grown in lactose broth (LB) medium at 30ºC with shaking, results showed that the recovery of polysaccharide was increased which accounted for 181 mg dry weight after lyophilization. Rajeshwari et al., (1995) found that LB was very effective for cultivation of *Arthrobacter* spp, and with the supplementation of 10 mM glucose, higher biomass yield and recovery of polysaccharide were recorded (20 gram weight bacteria). Most Gram positive bacteria are surrounded by a thick cell wall consisted of peptidoglycan decorated with surface proteins and polysaccharide-based polymer (Cole *et al.*, 2008). Most reports indicated the use of different method for the isolation of Gram positive cell wall, mostly by either physically or enzymatically procedures. The report of Wal *et al*., (1997) showed that due to the rigid walls surrounded the Gram positive coryneforms and *Bacillus brevis* cells were disrupted by exposing them five times to Frensh press between 15000 –20000 psi. While Cole *et al.*, (2008) used an N-acetylmuramidase enzyme (mutanolysin) for the preparation of Gram positive bacterial cell wall from streptococcus pyogenes.

The applied procedure for gram positive polysaccharide isolation can be considered as simple and easy to run procedure with significant yield of polysaccharide.

It was noticed that the suspension of dried bacterial cells in EDTA solution and the application of autoclaving result in destruction of bacteria cells and the denaturation of bacterial proteins. Following the centrifugation at 10000 rpm, two phases were separated, the upper phase (aqueous phase) contains the polysaccharide and nucleic acids and the lower phase contains denatured proteins and cell debris (Carlson *et al*., 1987). The aqueous phase was aspirated off and dialyzed against distilled

water in order to remove salts and other impurities (Weber *et al*., 1997). Most of polysaccharides were collected from the aqueous phase due to the aliphatic nature of polysaccharide, and their ability to be soluble in water (Vu *et al.,* 2009).

Reports showed that the yield of polysaccharide varies depending on the type of methods used, however, the of polysaccharide from Gram positive and negative bacteria ranged between 100-500 mg polysaccharide (Gerhardt *et al.,* 1981). Cruter et al., (1994) reported that 90 mg cell wall polysaccharide, free of protein, was obtained from 3 g of lyophilized *Lactococcus lactis* cells using extraction method depending on repetitive acid and alkaline hydrolysis.

3.3.1 Chemical Characterization of Polysaccharide:

Chemical characterizations of the crude polysaccharide extracted from *Arthrobacter* B1 was performed by estimating the carbohydrate contents according to Dubbois *et al*., (1956), on the standard curve of glucose, estimating the protein contents and the standard curve of bovine serum albumin according to Bradford, (1976).

Results indicated that the percentage of carbohydrates and proteins of crude polysaccharide were 8.9% and 1.9% respectively. Some methods for extraction of Gram positive polysaccharide revealed that the aqueous phase contains low proportions of protein associated polysaccharide (Vijayendra *et al.,* 2008). The cell wall of Gram positive bacteria contains a variety of polysaccharide branched which are covalently linked to peptidoglycan. The branches of the polymer also extended to bind the glycoproteins within the surface layer (s-layer) as a part of the 3-D structure of the Gram positive bacterial cell wall (Schaffer and Messner, 2005). Zhang *et al.,* (2001) indicated that Gram positive bacterial cell

wall associated proteins were removed by treatment with proteolytic enzymes include: pepsin, proteinase K and papain, followed by other chemical treatment. Nevertheless, detected amount of protein between 0.6 to 2.2% were analyzed via GC-MMS for the polysaccharide extracted from different species of *Eubacterium*. Polysaccharide separated from different Gram positive bacteria contain different forms of sugars like glucose, rhamnose, galactose (Van Calsteren, 2002)

3.3.2 Partial Purification of Polysaccharide:

Partial purification of *Arthrobacter* isolate B1 polysaccharide was carried out by using gel filtration chromatography on sepharose C1-6B column as described in item (2.2.7). When the polysaccharide was applied to the sepharose Cl-6B column previously equilibrated with 0.025 M phosphate buffer, 60 fractions were collected, and the polysaccharide was recovered by detecting the carbohydrate contents for each fraction at 490 nm, also the contents polysaccharide-associated proteins were detected at 280 nm. Figure (3-1) illustrates the separation of two peaks; one is large which mostly consisting of carbohydrates, and the other is small, moreover, both peaks were followed by protein peaks. This result comes in agreement with the study of Pier *et al*., (1978) who noticed that the presence of polysaccharide-associated proteins cannot be separated after one purification step.

In the recent study, the two distinct peaks revealing the presence of two polysaccharide molecules: a relatively board peak with a higher molecular weight and a small peak with a lower molecular weight, were subsequently separated following purification by gel filtration of polysaccharide from *Arthrobacter* isolate B1.

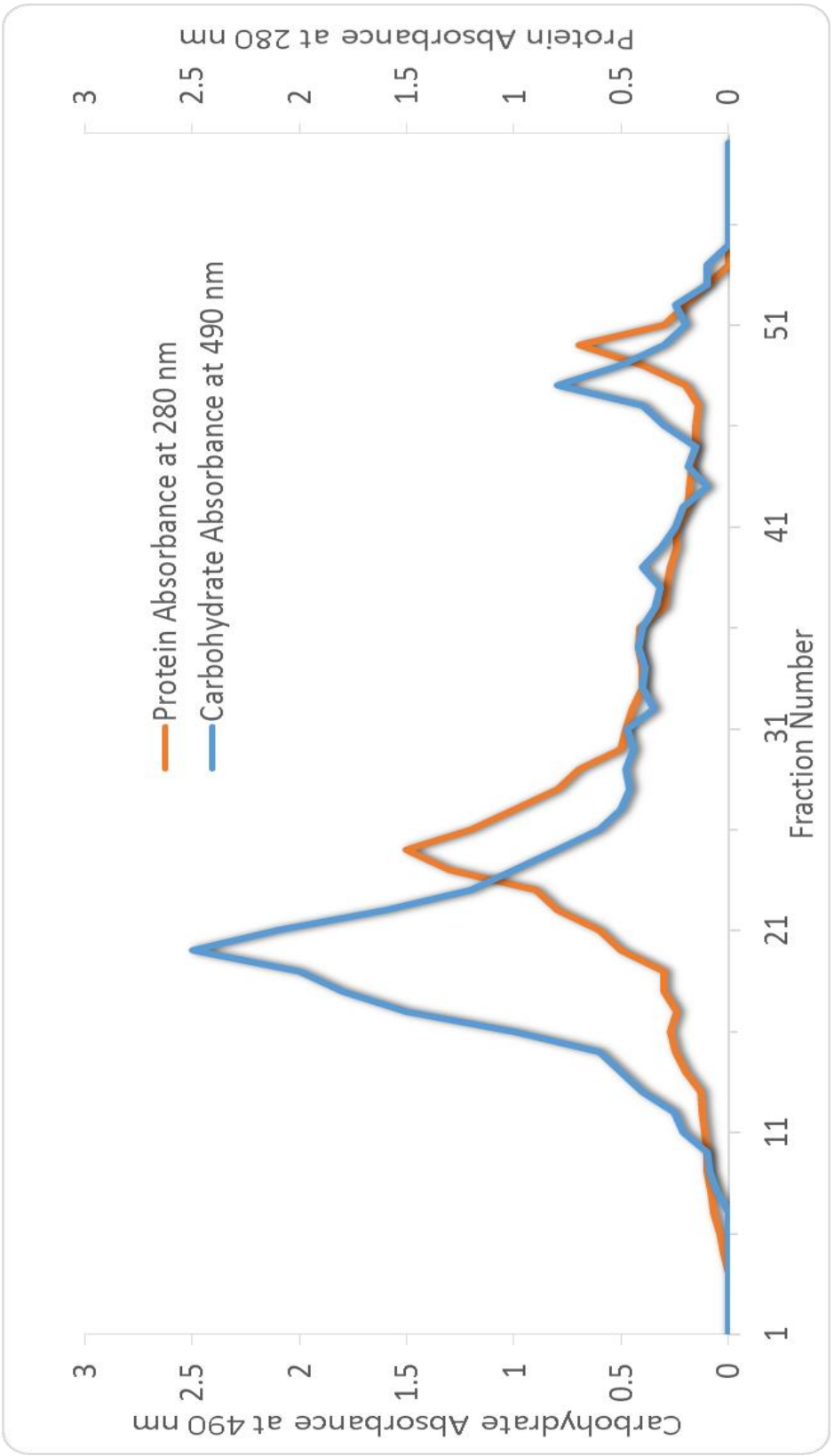

Figure (3-1) Purification of polysaccharide by gel filtration chromatography (Sepharose Cl-6B) column (3x50 cm) equilibrated with 0.025M phosphate buffer pH 7.2, flow rate 30ml/hour.

According to Grobben *et al.,* (1997), polysaccharide have different molecular weights, so the purification of *Arthrobacter* polysaccharide through sepharose Cl-6B that showed high molecular weight can be easily separated (fractions between 17-21) from lower molecular weight impurities.

Chemical analysis of the partially purified polysaccharide in the large peak (peak 1) was determined. Chemical analyses involved determination of carbohydrate, protein and nucleic acid contents for the active fractions from 17th to 21th appeared in the large peak. Results indicated that the percentage of carbohydrates was 25.1% in the large peak.

Related studies revealed the ability to obtain two fractions or peaks after loading the microbial polysaccharide on different chromatographic columns (ion exchange or gel filtration) in which the sugar content in the first large fraction was measured to be around 23% (Hamada and Slade, 1976; Ziaja *et al.,* 2011). Robjin *et al.,* (1996) reported that the overall percentage of carbohydrates present in *Lactobacillus* structure is polysacccharide 65%, while a percentage of 84% was reported by Vijayendra *et al.,* (2008) for the *Leuconostoc* polysaccharide. Such differences in calculations are generally attributed to the types of bacterial species from which polysaccharide was extracted, method of extraction and purification process.

Protein contents were also investigated and results showed that the protein percentage was 10.7% in peak 1. In addition the application of nucleases enzymes resulted in 0% of nucleic acids after gel filtration purification of polysaccharide.

3.4 Effect of Partially Purified *Arthrobacter* Polysaccharide on tumor cells:

Effect of *Arthrobacter* polysaccharide on tumor cells was examined by studying whether the bacterial polysaccharide affect the structure of tumor cell's DNA. For this purpose, uHepG2 cells were treated with colchicine (50 µg/ml), others with the partially purified polysaccharide (1 mg/ml), and a third group were not treated to be used as a control. Both treated and untreated cells were subjected to MNase digestion, followed by DNA extraction, electrophoresis and DNA banding pattern scanning by the gel documentation system.

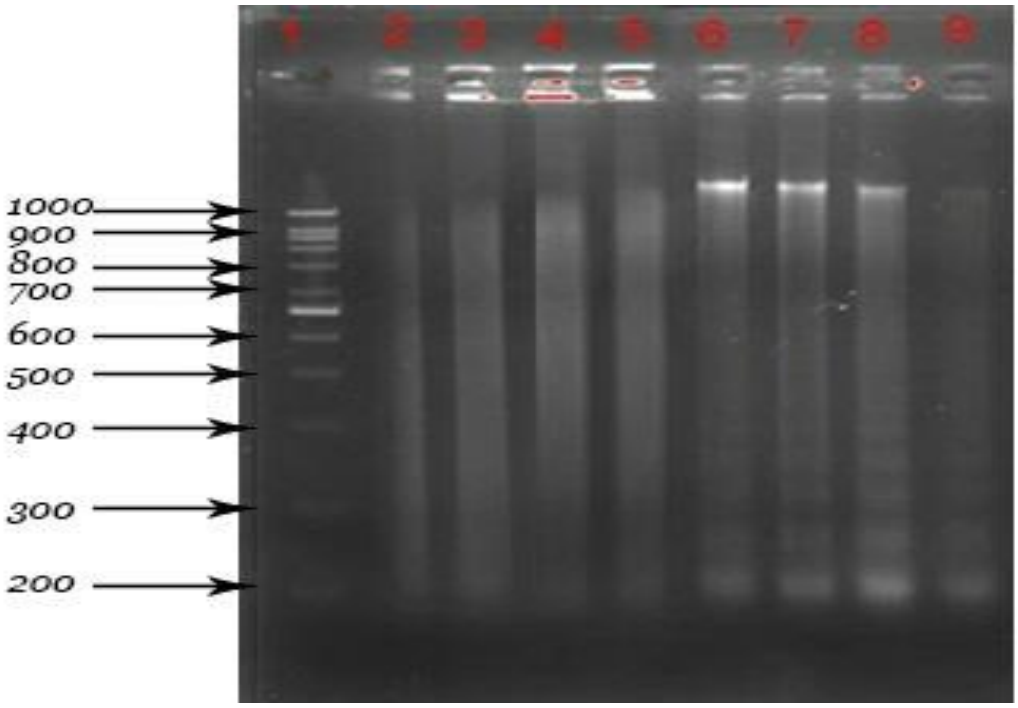

Figure (3.2). DNA extracted from tumor cell; lane 1 nucleosome ladder of MNase digested chromatine, lane 2 DNA from untreated cells undigested with MNase, lane 3 DNA from untreated cells digested with 5 µl MNase, lane 4 DNA from untreated cells digested with 7.5 µl MNase, lane 5 DNA from untreated cells digested with 10 µl MNase, lane 6 DNA from cells treated with colchicine undigested with MNase, lane 7 DNA from cells treated with colchicine digested with 5 µl MNase, lane 8 DNA from cells treated with colchicine digested with 7.5 µl MNase and lane 9 DNA from cells treated with colchicine digested with 10 µl MNase.

Figure (3.2) shows differences in DNA banding pattern between the untreated cells and those cells treated with colchicine. Results indicated that only intact nucleosomal DNA bands were separated on the gel for the untreated cells as compared with the banding pattern of the colchicine treated DNA, in which besides to intact nucleosomal DNA, mononucleosomes, dinucleosomes and oligonucleosomes were separated on the gel following electrophoresis process. In addition, in both cases the gradual increase in MNase concentration did not significantly affected the DNA digestion.

Colchicine is well known in inhibiting microtubule polymerization by binding to tubulin, thus inhibits the development of spindles, and eventually cells cannot move the chromosomes, instead, pull them apart and mitotically divided (Mendez and Stillman, 2000). Colchicine arrests the cell cycle at metaphase stage, this phase is characterized by disappearing of nuclear envelop and the DNA being more condensed and packed into chromosomes (Poirier *et al*., 2000). Metaphase chromosomes are widely used as a comparing control in many experiments related to nucleosome partitioning via MNase digestion (Stedman *et al*., 2004; Martinez-Garcia *et al*., 2010). Morever, the metaphase chromosomes contains very fewer non-histone protein in their structure in which the remaining metaphase scaffold mainly consists of topoisomerase which has a role in stabilizing the bases of DNA loops of metaphase chromosomes (Martinez-Garcia *et al*., 2010). However, recent study indicated the cytogenetic effect of colchicine (10 µg/ml) on the chromosomes of normal lymphocyte cells. The observations of the researchers were correlated between the exposure period and the length of chromosomes, in which the extended periods of exposure leads to more

short and condense chromosome. In contrast, short exposure period of several hours gave longer chromosomes (Koyani and Saiyad, 2011).

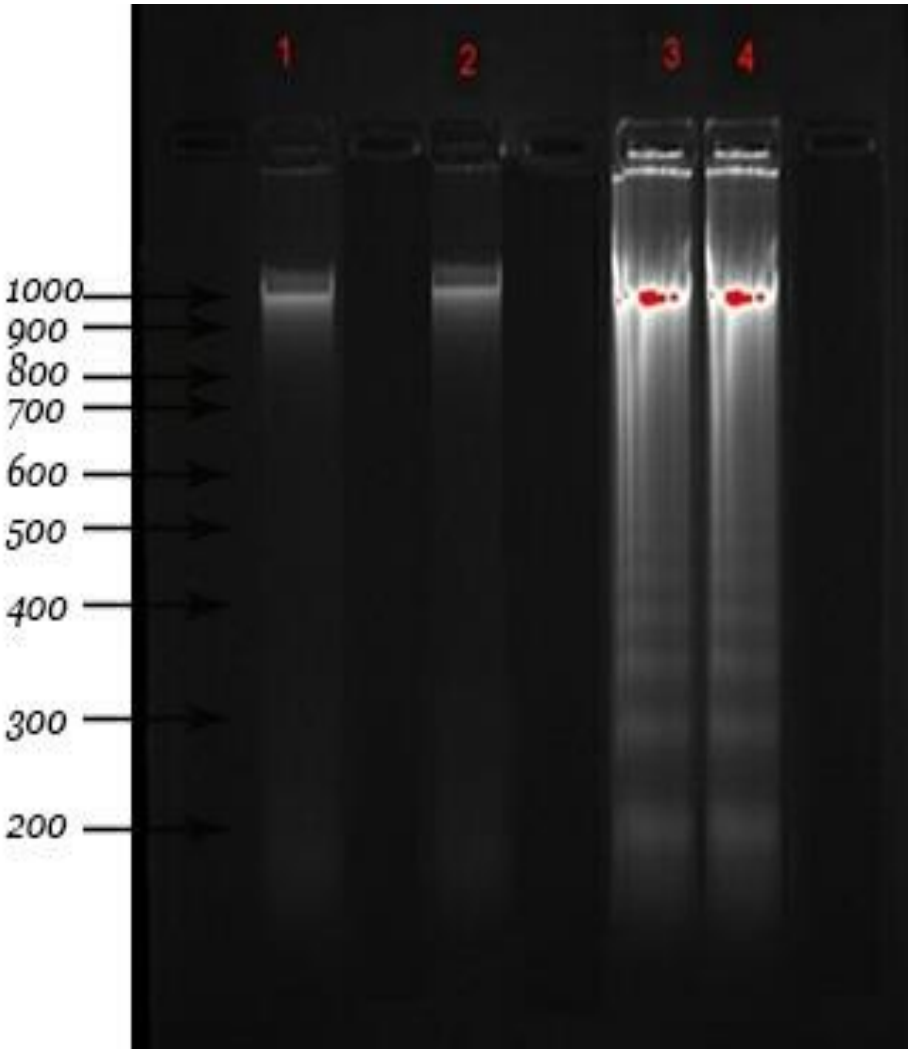

Figure (3.3). DNA extracted from tumor cell treated with *Arthrobacter* polysaccharide: lane 1 cells undigested with MNase, lane 2 cells digested with 5 µl MNase, lane 3 cells treated with 7.5 µl MNase and lane 4 cells digested with 10 µl MNase.

As in figure (3.3), the DNA banding pattern shows that *Arthrobacter* polysaccharide significantly affected the separation of nucleosomal DNA, in which mononucleosomes, dinucleosomes and oligonucleosomes were clearly separated from the intact nucleosomal DNA. Also by gradual increasing in the concentration of MNase, more separation was yielded as

compared with the cells treated with the colchicine. According to this result, it was suggested that *Arthrobacter* polysaccharide participated in tumor cell was lysed by induction of DNA fragmentation, which is a characteristic feature of the cell apoptosis.

Bacterial polysaccharide have been received an increasing attention in researchs through the last few decades. The roles of polysaccharides in biological systems were involved in several cellular processes, such as molecular recognition, cell development and differentiation, and cell–cell interaction. Many polysaccharides exerted potent antioxidant, anticoagulant, antithrombotic and antiviral activities and they are also known to inhibit some tumor development (Han *et al*., 2005; Yang *et al*., 2005).

Cell cycle is very important to the regulation of tumor growth. There are two Gaps of the cell cycle: the G1 and the G2 Gaps. The G1 cap decides whether the cells enter S stage via G1 stage, and it is the driving force of the cell cycle. The G2 Gap is the monitoring mechanism of the cell cycle; it ensures the high accuracy of DNA duplication and prevents cells with damaged or unduplicated DNAs from entering mitosis (M) stage (Yoshizawa et al., 1995).

Studies suggested that the flow cytometry analyses of polysaccharide from different sources causes' cancer cell cycle arrested at the G0/G1 or the G2/M check points (Zhang *et al*., 2011). In addition microbial polysaccharides can cause the tumor cells to undergo apoptosis by regulating signaling molecules such as NF-κB (Lin *et al*., 2005) besides to the change of Ca2+ concentration or can induced tumor cells to apoptosis. Moreover, polysaccharides can indirectly inhibit the growth of tumor cells by improving the immune functions or inhibiting DNA synthesis (Guo *et al*., 2005).

Shi *et al*., (2007) reported that treatment of human gastric carcinoma cells with polysaccharide being chemically modified with sulphate group induced cell apoptosis through DNA fragmentation which characterized by gel electrophoresis as distributed nucleosomal ladder of fragmented chromatin. It's very important to know that identification of nucleosome distribution and chromatin accessibility profiles provides a new set of measurements for the biological activity and/or antitumor activity of natural and chemical substances on the tumor cells.

Many studies defined the role of bacterial polysaccharide extracted from their cell wall as either antitumor agent or as immunomodular (Tanaka *et al*., 1989; Tzianabos, 2000), however little is known about how polysaccharide affect the genetic material and the gene expression of the tumor cells via chromatin remodeling which responsible for altering the position and density of nucleosomes and in turns it may allow genes to be transcriptionally active or not. Such strategy was adopted by treating immune cells with bacterial polysaccharides determine the genetic changes with respect to immune response, the resulted changes indicated that large number of affected promoter regions confirms that polysaccharide signaling in human macrophages induces a massive redistribution of nucleosome at many immune genes (Johannessen *et al*., 2013).

Chapter Four
Conclusions and Recommendations

CONCLUSIONS AND RECOMMENDATION

4.1 Conclusions:

1. Polysaccharide can be extracted from *Arthrobacter* B1 with high yield.

2. Two peaks were present after partial purification of *Arhtrobacter* B1 Polysaccharide using sepharose Cl-6B gel chromatography that showed the highest carbohydrate concentration.

3. Only intact nucleosomal DNA bands were separated on the gel for the untreated HepG2 cells as compared with the banding pattern of the colchicine and *Arthrobacter* B1 polysaccharide treated DNA, in which besides to intact nucleosomal DNA, mononucleosomes, dinucleosomes and oligonucleosomes were separated.

4. Nucleosomal DNA bands pattern of both untreated and colchicine treated DNA does not affected by the gradual increase in MNase concentration, but nucleosomal DNA bands pattern of DNA treated with polysaccharide in which the separation of nucleosomes became more clear by increasing the concentration of MNase.

4.2 Recommendations:

1. More studies about the effect of polysaccharide on the DNA of tumor cells by detecting chromosomal changes in gene expression via DNA microarray.

2. Studying the cellular mechanism by which the tumor cells respond to bacterial polysaccharides, which can be achieved by detecting receptors specific to polysaccharides.

3. Studing the cytotoxic activity of Gram positive *Arthrobacter* polysaccharide against different tumors *in vivo* and *in vitro*.

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الخلاصة

صممت هذه الدراسة للتحقق من تاثير متعدد السكريات البكتيري الموجب لصبغة كرام و المستخلص والمنقى جزئيا من بكتريا *Arthrobacter* sp. على نمط حزم الحامض النووي (الدنا) لخطوط الخلايا السرطانية في الزجاج. عشرة عينات تم جمعها من مواقع مختلفه في بغداد حيث تميزت هذه المواقع بكونها مناطق ورطبة, مظللة جزئيا وممزوجة او مغطاة بأوراق وسيقان نباتات ميتة. طبقا للفحوصات المجهرية والمظهرية والكيميوحيوية اظهرت النتائج بأن عزلة بكتيريه واحدة هي تنتمي لجنس *Arthrobacter*.

استخلاص متعدد السكريات من الغلاف الخارجي لبكتريا *Arthrobacter* B1 باستخدام طريقه تمزج بين تطبيقات الضغط والماء الساخن حيث كان عائد الاستخلاص هو 181 ملغم. كما اظهرت نتائج التحليل الكيميائي ان المحتوى الكاربوهيدراتي والبروتيني 15.9% و 1.9% على التوالي. تم اعتماد التنقية الجزئية لمتعدد السكريات بواسطة استخدام كروماتوكرافيا الترشيح الهلامي باستخدام هلام (Sepharose Cl- B6) وبعد التنقيه تم الحصول على قمتين و بعد تحديد الخواص الكيميائيه و التي تنطوي على تقدير المحتوى الكاربوهيدراتي والبروتيني اظهرت النتائج بأن القمة الاولى كانت ذات محتوى كاربوهيدراتي عالي 30.1% مع مكونات بروتينيه قليله 5.7%.

درس تأثير مستخلص متعدد السكريات و المنقى جزئيا من بكتريا *Arthrobacter* على خط الخلايا السرطانية HepG2 و ذالك بدراسة في ما اذا لمتعدد السكريات البكتيري تاثير في تركيب الحامض النووي للخلايا السرطانيه ام لا. تم تقسيم خط الخلايا السرطانية HepG2 الى ثلاث مجاميع: الاولى تمت معاملتها مع الكولجسين (50µg/ml), الثانيه تمت معاملتها مع متعدد السكريات المنقى جزئيا (1mg/ml) والمجموعه الثالثه لم تعامل وانما استخدمت كفاحص للمقارنة. كلا من الخلايا المعاملة وغير المعاملة تعرضت للهضم من قبل انزيم MNase ثم تم بعد ذالك استخلاص الحامض النووي والترحيل الكهربائي وفحص نمط حزم الحامض النووي باستخدام جهاز التدوين الهلامي.

اشارت النتائج الى انه فقط حزم الدنا النيوكليوسومي الكاملة قد فصلت في الهلام لدنا الخلايا الغير معاملة بالمقارنه مع نمط حزم الحامض النووي للخلايا المعاملة مع الكولجسين حيث بالاضافة الى الدنا النيوكليوسومي الكامل تم الحصول على فصل للنيوكليوسوم الاحادي, الثنائي والنيوكليوسوم المتعدد القليل في الهلام بعد عملية الترحيل الكهربائي. أن مثل هذا النمط

لم يتأثر بالزيادة التدريجية لتركيز MNase. من جانب اظهر نمط حزم الحامض النووي للخلايا المعاملة مع متعدد السكريات المستخلص من *Arthrobacter* B1 تأثيرا معنويا من خلال فصل نيوكليوسوم الحامض النووي الاحادي, الثنائي و المتعدد القليل بصورة واضحة عن نيوكليوسوم الحامض النووي الكامل حيث ان مثل مثل هذا التاثير كان ظاهرا بصورة واضحة مع زيادة تركيز MNase بالمقارنه مع الحامض النووي للخلايا المعامله بالكولجسين.

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا ۖ إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ ﴿٣٢﴾

صَدقَ اللهُ العَظِيم

سورة البقرة اية 32

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم

تاثير متعدد السكريات المستخلص المنقى جزئيا من بكتريا *Arthrobacter* spp. على نيوكليوسوم خطوط الخلايا HepG2

رسالة

مقدمة الى كلية العلوم-جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم

في التقانة الاحيائية

من قبل

نوار بهاء عبد الصاحب الربيعي

بكلوريوس تقانة احيائية, كلية العلوم, 2011

جامعة النهرين

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كانون الاول 2013