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Effect of *Bifidobacterium bifidum* Biosurfactant on HRT-18 Cancer Cell Line and Investigation of Gene Expression of *NOX4*

A Dissertation
Submitted to the Council of the College of Education for Pure Sciences/University of Kerbala as part of the Requirements for the Degree of Doctorate of Philosophy in Biology-Zoology

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صَدَقَ اللهُ العَلِيُّ العَظيمْ

سورة الشعراء - ايه ٨١

Dedication

To the best of all creation ... the most honorable being,
To the Prophet Muhammad, peace be upon him and his pure family,
To the one who devoted his life for me, bearing my burdens without hesitation,
To the one whose presence I wished for to share my joy … my role model, my beloved father, may Allah have mercy on him,
To the one for whom hands are raised in prayer … my dear mother, may Allah protect her,
To my pillar in life...
My dear husband,
To the flowers of love and the fruits of my heart … my sons
To every diligent seeker of knowledge,
I dedicate this humble effort.

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Supervisors Certification

We certify that this dissertation entitled "**Effect of *Bifidobacterium bifidum* Biosurfactant on HRT-18 Cancer Cell Line and Investigation of Gene Expression of *NOX4***" was prepared under my supervision at the a dissertation submitted to the council of the College of Education for Pure Sciences / University of Kerbala as a part of requirements for the degree of Doctorate in Philosophy Education / Biology-Zoology / Microbiology genetics-Cytotoxicity.

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Summary:

The current study involved the isolation and identification of *Bifidobacterium bifidum* and the evaluation of its ability to produce a Biosurfactant (BS). The (BS) was subsequently extracted and its effect was investigated on the Human Rectal Tumor cell line [HRT-18]. Then, the study assessed the gene expression of the NADPH Oxidase 4 gene (*NOX4*) in the same cell line following treatment with various concentrations of the extracted (BS), and the effects of these concentrations were compared to the chemotherapeutic FOLFOX.

The current study was conducted from the beginning of April 2024 to the end of January 2025 and included three main axes, the first axis was the isolation and diagnosis of bacteria and extraction of (BS) at the Al-Ameen Research Center/The Holy Shrine of Imam Ali (AS) Najaf/Iraq, the Biotechnology Research Center/Al-Nahrain University, and a specialized medical studies center in Baghdad/Iraq The second component focused on cytotoxicity Assays, while the third involved investigating the gene expression of *NOX4*. The latter two components were carried out at the Center for Natural Product Research and Drug Discovery, Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur.

Five *B. bifidum* isolates were obtained and screened for (BS) production. The extracted (BS) was lyophilized, and five concentrations (400, 200, 100, 50, 25) µg/ml were prepared.

Cytotoxic effects were detected using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (MTT) , activated cell line [HRT-18] were exposed to the five prepared concentrations for 24 hours at 37C°, the results showed cytotoxic effects of (BS) on the [HRT-18], a decrease in the number of cancer cells was observed with an increase in the inhibition rate with increasing

concentrations, with the highest inhibition rate at concentrations (400,200) µg/ml reaching (68.4, 61.3)% respectively.

While (BS) did not show acute cytotoxicity on the normal cells Human Dermal Fibroblasts neonatal [HDFn], the results showed significant differences $P \leq 0.0001$ in the calculation of the Inhibitory Concentration 50% (IC50) when cancer cells [HRT-18] were treated with (BS) 53.4 µg/ml, and in [HDFn] 93.2 µg/ml.

The results confirmed significant differences $P \leq 0.0001$ between (BS) and FOLFOX in the [HRT-18] at concentrations between (400, 25)µg/ml, while the results showed significant differences between (BS) and FOLFOX in [HDFn] at concentrations (400, 200)µg/ml and no significant differences at concentrations (100, 50, 25)µg/ml.

Based on MTT results, High Content Screening (HCS) was conducted on [HRT-18] cells using (BS) concentrations between (200, 25)µg/ml, with comparisons to untreated cell controls and (FOLFOX) 200 µg/ml.

The results of the (HCS) confirmed significant effects on most cellular indicators with an increase in the concentration of the (BS) on the [HRT-18], particularly at concentrations of (200, 100)µg/ml , compared to the negative control , the results also indicated no significant difference between the effects of (BS) and FOLFOX at a concentration of 200µg/ml.

The results of the Caspase-9 and Caspase-8 assays showed that Caspase-9 exhibited a significant increase compared to the negative control, on the other hand, Caspase-8 showed lower activity, compared to Caspase-9, suggesting that the main pathway in the opoptosis is the intrinsic pathway, mediated by Caspase-9. Moreover, the results revealed no significant differences between the effects of the (BS) and the FOLFOX at the concentration of 200µg/ml, when compared to the negative control in both apoptotic pathways.

The results of the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) anti-Oxidant assay showed no significant differences between (BS) and Ascorbic acid when treated the [HRT-18] cell line with a small significant difference at a concentration of 50µg/ml.

The results of the Reactive Oxygen Species (ROS) assay showed that there were significant differences and an increase in ROS production at concentrations (200, 100)µg/ml, and the results showed no significant differences between the effect of (BS) and FOLFOX at a concentration of 200µg/ml, compared to the negative control.

The results of the investigation of the gene expression of *NOX4* gene in the [HRT-18] after treating it with different concentrations of (BS) using the Quantitative Reverse Transcription Real-Time PCR (RT-qPCR) assay showed , (BS) has cytotoxic effects with significant differences $P \leq 0.0001$ in the gene expression of *NOX4* gene in the [HRT-18] after treatment with concentrations (200,400)µg/ml, compared to the negative control, while at concentrations (100, 50, 25)µg/ml no significant differences appeared in gene expression, meaning that with increasing concentrations of (BS), the gene expression of the anti-cancer *NOX4* gene increases.

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List of Abbreviations

Abbreviations	Meaning
BE	Bioemusifiers
BS	Biosurfactant
HDFn	Cell Fibroblast
CMP	Cell Membrane Permeability
CC	Cytochrome C Releasing
[DLD-1]	Derived from Duke's Type D Colorectal Adenocarcinc
DPPH	Diphenyl Picrylhydrazyl Radical Scavenging
E24	Emulsification Index
EDTA	Ethylene Diamin Tetraacetic Acid
FBS	Fetal Bovine Serum
HCS	High Content Screening
[HCT-15]	Human Colon Tumor-15
[HCT-8]	Human Colon Tumor-8
[HRT-18]	Human Rectal Tumor-18
IFT	Interfacial Tension
MTT	Methylthiazolyldiphenyl-Tetrazolium Assay
MMP	Mitochondrial Membrane Potential
PBS	Phosphate – Buffer Saline
PCR	polymerase Chain Reaction
ROS	Reactive Oxygen Species
RT- qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
RPMI-1640	Roswell Park Memorial Institute Medium 1640
SFM	Serum Free Media
SFT	Surfactant Tension
SG	SYBR Green Stain
TNI	Total Nuclear Intensity
VCC	Viable Cell Count

Chapter One

Introduction

1-Introduction

Cancer is one of the most important diseases that affect humans and includes more than 100 different diseases that can occur in any part of the body, one of the distinguishing characteristics of cancer is the rapid growth and proliferation of abnormal cells, which have the ability to invade nearby tissues and spread to other organs through blood vessels in a process known as metastasis, these widespread metastases are a primary cause of death (Sherman *et al*.,2022).

Malignant tumors affecting the human large inAssayine, in all their types, rank third in occurrence compared to other types of malignant tumors, it ranks fourth as a leading cause of death worldwide. It is commonly found in humans, with an occurrence rate of 58% in men and 47% in women over the age of 40 in countries with a high incidence of the disease (Lui *et al*.,2021).

In Iraq, colorectal cancer is a common disease among the elderly, ranking second with an incidence rate of 7.3%, average 6.8 hundred thousand people, incidence rate compared to other types of cancer in the country, in addition its incidence is also increasing just like other types of tumors (Iraqi Cancer Board,2022; American Cancer Society,2023).

Bifidobacterium bifidum was identified in the last century, and numerous studies have highlighted its therapeutic properties that contribute to improving human health. These bacteria are found in the inAssayines, with Bifidobacterium spp making up 99% of the large inAssayine's microbial content. They are mainly located in the inAssayinal lining and vaginal canal, helping to digest fibers, prevent infections, and produce several secondary metabolic compounds used in treating microbial infections (Henry,2023).

It produces important compounds such as vitamin B and healthy fatty acids, it is a type of beneficial bacteria known as probiotics, fermented foods are a good source of this bacteria, and many studies have proven that they are effective in

preventing digestive diseases, boosting immunity, and treating tumors, they are also helpful in eliminating foodborne pathogens, such as Salmonella, which causes diarrhea (Sakanaka *et al*.,2019).

Biosurfactants are biological substances produced by various microorganisms, including *B. bifidum* bacteria, they work by reducing surface tension and are considered antimicrobial and anti-adhesive agents (Chen *et al*.,2021).

To investigate the cytotoxic effects of the (BS), researchers have developed a technique that facilitates the study of therapeutic effects *in vitro* against different types of cancer cells ,this technique is called tissue culture, and cancer cell lines are used ,through this method , the cytotoxic effect of compounds on cultured cells can be evaluated the researcher has the ability to control the environmental conditions for cultivating and preparing cancer cells, as well as the exposure time to them (Marcelino *et al*.,2020).

NADPH Oxidase 4 gene (*NOX4*) is a human gene and is a member of the NOX enzyme family that produces (H_2O_2) and has an important role in cell proliferation, migration, and programmed death (apoptosis) (Dewa *et al*.,2024). The *NOX4* gene regulates certain beneficial adaptive responses mediated by reactive oxygen species (ROS) and is located on chromosome 11 with a precise locus of 11q14.3, causing cancer cell death by increasing gene expression. (Xirouchaki *et al*.,2021).

1-1-Aim of the Study

The aim is to find a safe biological treatment as an alternative to chemotherapy, which is more effective, less expensive, and has fewer side effects on normal cells, through the following axes:

1. To sample collect, isolation, and diagnosis of *Bifidobacterium bifidum.*

2. To extract Biosurfactants (BS) from *B. bifidum*.

3. To prepare different concentrations of (BS) in µg/ml (400, 200, 100, 50, 25)

4. To activate the cells of the colorectal cancer cell line [HRT-18], performing the MTT cytotoxicity assay and investigating the effect of different concentrations prepared from (BS) on the colorectal cancer cell line [HRT-18] and comparing it with the normal cell line [HDFn] and the effectiveness of chemotherapy. FOLFOX.

5. To conducte a high-content HCS assay to evaluate the cytotoxicity of (BS) in colorectal cancer cell line [HRT-18] for concentrations that will show an effective result in the MTT assay and compare it with the effectiveness of FOLFOX chemotherapy to identify the mechanism of cancer cell death.

6. To conducte a Caspase-9 and Caspase-8 assay to investigate which of the two caspase pathways is responsible for the programmed death of the colorectal cancer cell line [HRT-18] when treated with different concentrations of (BS) extracted from *B. bifidum* bacteria.

7. To conducte a (DPPH) Scavenging A activity assay to identify the extent of oxidation (free radical scavenging) when treating the cancer cell line [HRT-18] with different concentrations of the (BS).

8. To show a Reactive Oxygen Species (ROS) assay to determine the level of oxygen and to evaluate reactive oxygen species and their role in cancer development.

9. To investigation of gene expression of *NOX4* gene in Colorectal Cancer cell line after treatment with BS extracted from *B. bifidum* by Quantitative Reverse Transcription Real-Time PCR (RT-qPCR) Analysis.

Chapter Two

Literature Review

2- Literature Review

2-1- Colorectal Cancer

The appearance of cancer in any organ of the human body is essentially a disturbance in the structure and activity of DNA in some cells, which causes a defect in the functions of chromosomes and genes that control cell functions and growth. The cancer cell acquires the characteristic of immortality, meaning the cancer cell continues to divide and multiply. Continuously without control, and when the number of cancer cells increases significantly, some of them are destroyed by this crowding, while others clump together, forming a mass of excess tissue called a tumor , which can be malignant or benign (Al-Khalili,2022).

When normal cells turn into cancer cells in an organ, the cells lose their normal characteristics and function, and the body loses the ability to control their accelerated reproduction and growth (Diaz *et al*.,2013). One of the most important cancers that appear in families under the influence of genetic factors is colorectal cancer, Cancer genes can be detected in the genes of a person whose family members have been exposed to colorectal cancer (Wu *et al*.,2013).

According to the World Health Organization (World Health Organization) 2014, Colorectal Cancer (CRC) is one of the most common types of cancer that can affect all ages and both sexes, which originates from the large inAssayine and may cause death if not diagnosed early and neglected treatment, and colorectal cancer ranks third in incidence from other cancers worldwide with the highest incidence in developed countries and it is estimated that by 2035 about 24.4 million new cases will be diagnosed colorectal cancer (Siegel *et al*.,2014).

Colorectal Cancer has increased in recent decades, accounting for 10% of cancer deaths, as its symptoms are not felt in the early stages without regular screening, and it is often diagnosed at advanced stages with limited treatment options (Kuipers *et al*.,2015).

Colorectal Cancer has become the second and third most common cancer in females and males, respectively, in western and developed countries (Kuipers *et al*.,2015). This increase is attributed to an aging population, poor dietary habits, smoking, obesity, low physical activity, and excessive alcohol consumption (The (Organization for Economic Cooperation and Development-OECD,2015).

In Iraq, several descriptive studies have been conducted on (CRC) and showed that the incidence of colorectal cancer was low but started to increase in recent years (Al Dahhan & Al Lami,2018). These Iraqi descriptive studies of (CRC) included patient gender, age, and symptoms (Al-Bayati & Jasim,2009 ; Al- Janabi *et al*.,2015).

Colorectal Cancer is a type of cancer that affects the colon and spreads to the rectum (large intestine) and can cause significant damage and death (Al-Janabi *et al*.,2015). Many studies have revealed that the risk of colorectal cancer increases with age and most cases occur in people over the age of 50 (Steele *et al*.,2014). The risk (CRC) can be reduced by eating a healthy diet, quitting smoking, limiting alcohol consumption, and exercising, and regular checkups are extremely important for early detection of the disease (Kuipers *et al*.,2013).

The American Cancer Society confirmed that in 2023, colorectal cancer will be the second leading cause of cancer deaths in the world, and it is estimated that there will be more than 1.9 million new cases of colorectal cancer and more than 930,000 deaths from colorectal cancer worldwide in the next few years (Islami *et al*.,2017; Jemal *et al*.,2010). Men are more likely to be affected than women in most parts of the world, with high incidence in North America and Europe and low incidence in South Central Asia and Africa (Torre *et al*.,2012).

The highest incidence and mortality rates were observed in Europe, Australia and New Zealand, while the highest death rates were recorded in Eastern Europe, the Colorectal Cancer burden is projected to increase to 3.2 million new cases per

year, an increase of 63%, and 1.6 deaths per year, an increase of 73%, by 2040 (Siegel *et al*.,2015).

Colorectal Cancer rates have declined in developed countries due to advances in early diagnosis methods, as the prognosis of Colorectal Cancer varies depending on the stage of the disease at the time of diagnosis (Chen *et al*.,2017). The likelihood of recovery is higher for people with cancer detected in the early stages compared to people with cancer detected in the late stages (Steele *et al*.,2014). Early correct diagnosis, correct treatment, and regular care and follow-up are important in achieving a cure (Neagoe *et al*.,2004).

2-2- Symptoms of Colorectal Cancer

The most common symptoms of Colorectal Cancer are weakness or fatigue, lack of appetite, poor health for no apparent reason, changes in bowel habits such as increased diarrhea or constipation, rectal bleeding with blood in the stool, nausea or vomiting, persistent abdominal pain such as cramps and gas, and a feeling of not completely emptying the abdomen when defecating , persistent bloating, marked weight loss for no apparent reason, lack of appetite, deteriorating general health and low iron levels, and many patients are often asymptomatic in the early stages of the disease (Jiang *et al*.,2013).

2-3- Colorectal Cancer Types

Colon Cancer is a type of cancer that develops from the inner lining of the large inAssayine (colon), and in many cases, colon cancer and rectal cancer coincide at the same time (Koch *et al*.,2001). Scientists were able to identify four types of Colon Cancer, which will greatly help in its diagnosis and treatment. Colorectal Cancer is divided depending on the type of cells from which it developed, as 95% of Colorectal Cancers are adenocarcinoma, the most common type that developed from epithelial cells of the mucous membrane lining the colon and rectum (Stanojević *et al*.,2009). The types of Colorectal Cancer are :

1. Primary Colorectal Lymphomas
2. GastroinAssayinal Stromal Tumors
3. Leiomyosarcomas
4. Carcinoid Tumors

1- Primary Colorectal Lymphomas

Primary Colorectal Lymphomas are Colorectal Cancers that involve lymph nodes and immune cells in general (Aisenberg *et al*.,2000). The World Health Organization (WHO) has identified several histological subtypes of lymphoma and confirmed that all types of these nodular lymphomas may develop in the GI tract, but the two main types that appear in more than 90% of cases are B-cell Non-Hodgkin's Lymphomas (NHL) and MALT lymphoma (MALT) NHL or Mucosa-Associated Lymphoid Tissue lymphoma (MALToma) (Khalil *et al*., 2014).

2- GastroinAssayinal Stromal Tumors

It is one of the rare colorectal cancers with an incidence of approximately 10% (Kays *et al*.,2018). It can occur at any age and the incidence rate between the sexes is the same (Kim *et al*.,2005), occurring in the cells of the colon wall that stimulate the colon muscular tissue to move food (Casali *et al*.,2018). The exact cause of these types of gastroinAssayinal tumors is not known, but some studies and the American Joint Committee-AJCC (2017), have stated that some are caused by the transmission of specific genes from parents to children (Jumniensuk & Charoenpitakchai,2018).

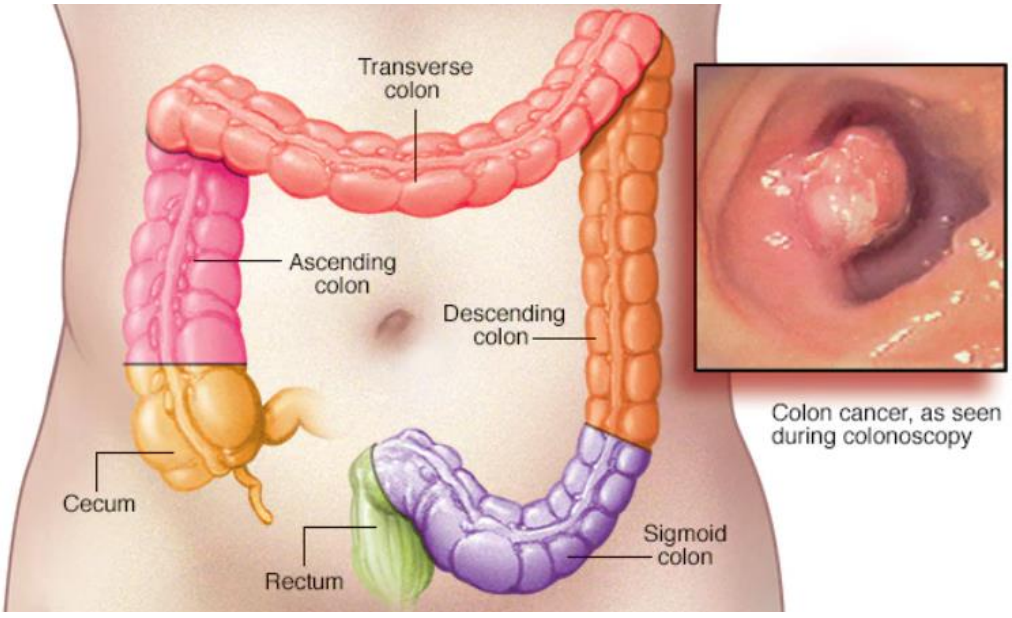
3- Leiomyosarcomas

Leiomyosarcoma is a rare type of cancer that occurs in smooth muscle and soft tissue. Smooth muscle tissue is found in many parts of the body such as the digestive system, urinary system, blood vessels, and uterus (Serrano & George, 2013). Most often the growth occurs in abnormal cells so that these cells begin to

multiply rapidly and invade and destroy neighboring normal body tissues (Katz & DeMatteo,2008).

4- Carcinoid Tumors

It is a type of cancer that belongs to a subgroup of neuroendocrine tumors and is slow-growing and occurs in different areas of the body, mostly in the gastroinAssayinal tract (stomach, appendix, small inAssayine, large inAssayine, colon and rectum) or the lungs, in cells that produce certain hormones within these organs (Mitchell,2005). Carcinoid tumors do not show symptoms until the advanced stages of the tumor by releasing excessive amounts of hormone-like substances (such as serotonin) into the body causing a carcinoid syndrome to produce symptoms such as diarrhea, redness of the skin color and a warm sensation in the face and neck causing what is known as dermatosis (Gala & Chung,2011), women are more likely than men to develop carcinoid tumors, and carcinoid tumors can be treated with surgery or medication depending on the stage of the tumor (Cress *et al*.,2006). Pictur (2-1) shows the detection of colon cancer and polyps by performing a comprehensive colonoscopy using a colonoscopy screening device.



Pictur (2-1) Locations of Colonrectal Cancer Seen by Endoscopy

Mayo Expert (2022) for Medical Education and Research

2-4- Causes of Colorectal Cancer

There are no specific causes of colon and rectal cancer, and it may occur under the influence of several different factors, and the true causes of all types of cancer are still largely unknown. But there are some causes that may lead to this, which are exposure to chemicals and carcinogens, radioactive materials and radiation, hormonal or genetic imbalance, heredity and family history of the disease, which may produce a mutation in the DNA found in the cells of the colon or rectum. This mutation causes cells to lose their ability to control the speed of growth and division, causing a defect in the nature of the body's functions as a result of the random growth of cells that accumulate and form a tumor. These cancer cells can destroy the normal tissue adjacent to the colon after a period of time, and may move to other parts of the body (Ervik *et al*., 2023).

2-5- Risk Factors

Numerous studies have confirmed that factors that increase the risk of colorectal cancer include :

1. Aging Factor

Colon cancer can occur at different ages, but most studies have emphasised that people over the age of 50 are more likely to develop the disease than others for reasons that are not yet clear (Steele *et al*.,2014).

2. black Race

Black Americans are more likely to be affected as indicated by the source (Bray *et al*.,2002).

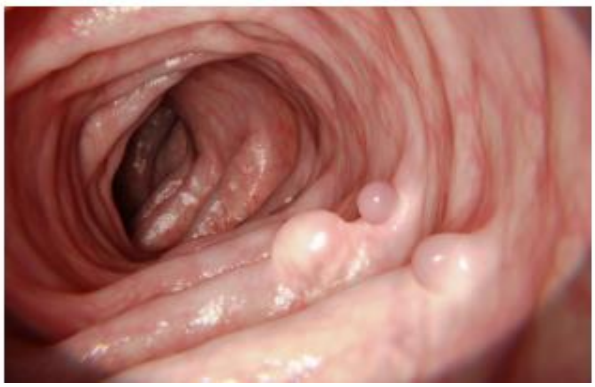
3. History of Previous Family Infections

Having a family history of colorectal cancer increases the likelihood of developing the disease if family members have had it since birth, and the risk is even higher if one or more family members have had colon or rectal cancer (Beebe *et al*.,2017).

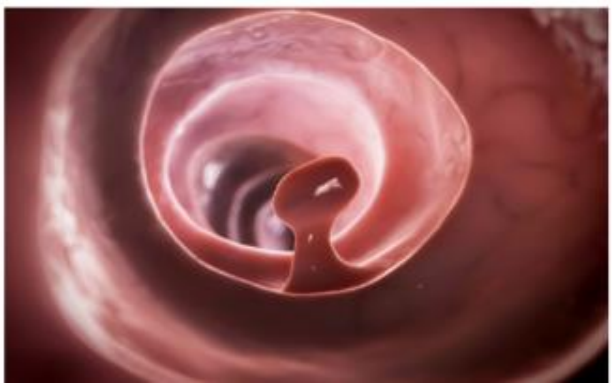
4. Familial Genetic Factors and Inherited Syndromes

These factors can cause colorectal cancer such as Lynch Syndrome, Lynch Syndrome is caused by a genetic mutation that causes colorectal cancer in 70 to 80 per cent of people who have the mutation and Lynch Syndrome patients develop colorectal cancer, often before the age of 50, and may have an increased risk of developing other types of cancer, especially endometrial cancer and ovarian cancer, and an increased risk of stomach cancer, small bowel cancer, and colon cancer. They are more likely to develop colorectal cancer before the age of 50 and may be at increased risk of other cancers, particularly endometrial and ovarian cancers, as well as stomach cancer and cancers of the small inAssayine, bile ducts, kidneys, and ureters (Dobre *et al*. ,2015; Obuch *et al*.,2016).

MUTYH (familial adenomatous polyposis) and Colorectal polyps are tumours that grow in the wall of the colon and rectum and come in different forms, including polyps that are elevated and resemble mushrooms as in Pictur (2-2)A or polyps that have a wide base and a rounded top as in Pictur (2-2)B , a rare genetic disorder that can cause colorectal cancer and is caused by genetic mutations in the MUTYH gene. Research has shown that more than half of people with this syndrome develop rectal cancer by the age of 60, and are at increased risk for other types of cancer, such as gastroinAssayinal and bone cancers, ovarian, bladder, thyroid, and skin cancers (Obuch *et al*.,2016).



Pictur (2-2)B Colorectal polyps



Pictur (2-2)A Colorectal polyps

National Comprehensive Cancer Network -NCCN (2024)

Colorectal Cancer Screening Guidelines)

5. Diabetes Mellitus or Insulin Resistance

People with diabetes or insulin resistance have a higher risk of developing and recurring colorectal cancer (Budzynska *et al*.,2018; Chubak & Ziebell,2018).

6. InAssayinal Inflammatory Diseases and Benign Tumors

Patients with inflammatory bowel disease, Crohn's disease or ulcerative colitis are also at higher risk in terms of the size of the affected area of the inAssayine or rectum, the length of the infection depending on the patient's age at the time of the disease (Smith *et al*.,2004), as well as the person's previous infections. People who have previously had colorectal cancer or certain types of polyps are at higher risk of developing the disease again (Al-Saigh *et al*.,2019).

2-6- Methods of Treatment

Several methods have been discovered to treat colorectal cancer depending on the type of tumour, tumour location, tumour stage, tumour spread and the general condition of the patient in the presence of other diseases. Treatment is either surgery, chemotherapy, radiation therapy or modern methods of biological therapy, which is still under research and development (Rodriguez,2023). They are as follows:

1. Surgery Therapy

Colon Cancer treatment is most often treated with surgeries and the surgery depends on the location and size of the cancerous tumour, where partial resection is done by removing the affected part with simple removal of some healthy parts surrounding the affected area, and surgery is the only solution in the initial stages of colon cancer, and lymph nodes close to the tumour are also removed during surgery to detect the stage of the disease, and the lymph nodes are examined by a microscope to detect the presence of cancer cells (Goldman, 2020).

Lymph nodes near the tumour are removed during surgery to detect the stage at which the disease has reached and the lymph nodes are examined with a microscope to detect the presence of cancer cells, if the glands are infected, this is

an indication that the disease has spread to other areas of the body and that the patient needs additional treatment after surgery (Goldman,2020).

2. Chemotherapy

Chemotherapy is several chemical drugs that destroy cancer cells and can be taken orally or injected intravenously, each of which treats a specific type of cancer with specific doses and a specific schedule, and may combine more than one drug for treatment depending on the disease state (Niederhuber,2020).

Chemotherapy is given to Colon Cancer patients in several ways, either before surgery to shrink the tumour and be able to remove it without complications, or it is given after surgery and tumour resection to remove any cancer cells remaining after surgery, or it is given as a palliative treatment if the cancer has spread to other areas of the body and cannot be eliminated by surgery, and the purpose of chemotherapy in this case is to relieve symptoms and tumours, and among the chemotherapies for colon cancer are (American College of Rheumatolog - ACR, 2019) :

- FOLFOX (Folinic acid, Fluorouracil and Oxaliplatin)
- FOLFIRI (Folinic acid, Fluorouracil and Irinotecan)
- CAPOX (XELOX) (Capecitabine and Oxaliplatin).

Chemotherapy is resorted to after surgery in the late stages of colon cancer, with a high likelihood of cancer recurrence, while in the case of early stage colon cancer, there is often no need to use chemotherapy, and chemotherapy may require eight treatment cycles in periods ranging between 2-3 weeks for each cycle, and the success rate of chemotherapy for colon cancer depends on the stage of the disease and the extent of its spread, and because chemotherapy is given in conjunction with other treatments for colon cancer, it is difficult to determine the effectiveness of each treatment without a combination (Warner, 2019).

3. Radiotherapy

Radiotherapy is done by using high-energy rays directed towards cancer cells to eliminate them and prevent their spread, and the goal of radiotherapy is similar to surgical treatment where radiotherapy is a localised treatment, i.e. it has an effect on cancer cells in the specific treated site only, and the doctor may resort to radiotherapy before surgery sometimes to shrink the size of the tumour, which helps make it easier to remove. Sometimes radiation is given before surgery to shrink the size of the tumour, which helps make it easier to remove, and radiation is also given after surgery to eliminate cancer cells that could not be removed during surgery, and radiation may be used to relieve pain and other complications in the patient when surgery cannot be performed to remove the tumour (Hall,2021; NCL,2023).

4. Biological therapy

It is called biologic or immunotherapy. It is a treatment that harnesses the body's natural immunity and lines of defence to fight and eliminate cancer cells. Biologic cancer therapy aims for the immune system to recognise and kill cancer cells and usually has fewer toxic side effects than other methods of cancer treatment (National Cancer Institute – NCL,2023).It may be used as a synergistic treatment with chemotherapy after surgery (Papiez,2021).

There are many types of biological therapy, including cancer vaccines, cytokine therapy, gene therapy, oncolytic virus therapy and other biotherapies. Cancer biotherapy is a very active area of cancer research and studies are still ongoing to uncover more information about this therapy (Niederhuber,2020).

2-7- Colorectal Cancer Cell Line [HRT-18]

The cancer cell line in general is the extraction and isolation of cancer cells from the living body and growing them in special vessels in the laboratory outside the body by providing favourable environmental conditions for them to continue to grow, divide and multiply for many years, then the cells are kept in liquid

nitrogen and continue to grow them in special culture media and transfer them from time to time to new vessels to be ready and at the service of scientific research when the researcher requests them (Hanahan & Weinberg,2000).

The process of developing a cancer cell line is so difficult and delicate that most scientific centres and laboratories have been unable to develop any cancer cell line due to the difficulty of providing the appropriate conditions for it, and the process of developing and creating them has been limited to developed countries that have established the International Bank for Global Lines (Hanahan & Weinberg, 2011 ; Gillet *et al*.,2013).

Studies examining the morphological changes in human colon cancer cell lines revealed that there are four types of human colon cancer cell lines: Human Colon Tumor-8 [HCT-8] , Human Rectal Tumor-18 [HRT-18] , Derived from Duke's Type D Colorectal Adenocarcinoma [DLD-1] and Human Colon Tumor-15 [HCT-15] , with an epithelial morphological pattern consisting of repetitive round cells and suffering from a deficiency of α-catenin , α-catenin is an important protein in cells that provides communication between the actin cytoskeleton and the cell membrane (Shinji *et al*.,2025).

Using DNA fingerprinting of colon cancer cell lines, it was shown that the four colon cancer cell lines have identical common genetic origins (Vermeulen *et al*.,1998). Findings from several studies suggest that these cells harbour a genetic mutation capable of losing α-catenin recurrently (Rimm *et al*.,1995).

The [HCT-8] cancer cell line is highly phenotypically and genetically identical to the [HRT-18] cancer cell line and it is suspected that the HCT-8 and [HRT-18] colon cancer cell lines are the same (Nelson-Rees,1978).

[HRT-18] also known as [HCT-8], is a vital cell line for exploring the underlying mechanisms and potential therapies of colon adenocarcinoma, derived from human colon tissue, [HRT-18] occupies an important position in colorectal

cancer research. Scientists take advantage of this line to gain insight into tumour biology, explore molecular pathways, and develop new therapeutic strategies, making it useful in the development of adenocarcinoma research and colorectal cancer in general (Tompkins *et al*.,1974).

[HRT-18] cells were isolated from the large inAssayine (Biochrom Berlin, Germany) of a 67-year-old adenocarcinoma patient and maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (PAA) and preserved for subsequent use in cancer research, toxicology and human cytology (Tompkins *et al*.,1974).

Although advances in genomics over the past decade have opened new horizons for translational research and allowed direct evaluation of clinical samples, there is still a need for reliable preclinical cancer cell line models to Assay therapeutic strategies. Human cancer-derived cell lines are the most commonly used models to study cancer biology and Assay hypotheses to optimise the efficacy of cancer therapy based on recent studies that have fuelled this debate (Chen *et al*.,1995).

2-8- Mechanisms of Cell Death

Programmed cell death (PCD) is an essential process for normal development and for many diseases such as cancer, autoimmune syndrome and liver disease by balancing cell division with cell death (Jung *et al*.,2020), the mechanisms of cell death are Apoptosis, Autophagy and Necrosis (Mery *et al*.,2017; Rode,2008).

1-Apoptosis

It is a natural mechanism of programmed cell death in mammals caused by phenotypic cellular changes including blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation and global mRNA decay (Karam & Jose,2009).

Programmed death plays an essential role in the development and balance of cells in an organism by eliminating damaged and excess cells and balancing the

production of new cells with cell death, which in turn will maintain the balance of cell numbers in tissues (Katherine,2013).

Apoptosis is a complex and regulated process that occurs through one of two main pathways (internal or external), In the internal pathway, the cell kills itself when it senses cell stress, and in the external pathway, the cell kills itself due to signals from outside the cell, so both pathways work to induce cell death through the activation of Caspases or Proteases (Koff *et al*.,2015).

2- Autophagy

Autophagy is a way for the cell to conserve energy sources in case of lack of oxygen or nutrients by producing amino acids needed to sustain life, disposing of damaged organelles that have lost their function, and getting rid of viruses and microbes that attack cells (Galluzzi & Green,2019). Autophagy also helps maintain the genetic makeup of cells, which helps prevent several diseases such as cancer, immune diseases, neurological diseases, heart and liver disease, and diabetes (Weiskirchen & Tacke,2019).

Studies have indicated that cellular autophagy has three forms: total autophagy, partial autophagy, and autophagy by intermediate proteins called Chaperon-mediated autophagy. Each of these forms has a method for cellular autophagy, but they all perform the same function (Badadan,2012).

Research has indicated that some chemotherapy and radiotherapy treatments for cancer induce cell autophagy, causing resistance to treatment and survival, requiring the use of other types of treatment that inhibit autophagy and change the elimination pathway to programmed death (Perez *et al*.,2019).

3- Necrosis

Necrosis is an uncontrolled episodic form of cell death that lacks the key signalling event and lacks the characteristics of apoptosis. This form may be best suited for damaged cell death caused by severe physical damage such as hyperthermia (Duprez *et al*.,2009), where the necrotic cell exhibits cytoplasmic hypertrophy, plasma membrane rupture, expansion of organelles (mitochondria, endoplasmic reticulum, Golgi apparatus and chromatin condensation) and caspase activation (Kroemer *et al*.,2005).

2-9- Role of Mitochondria in Apoptosis

Mitochondria have a role in apoptosis. Several molecules secreted into the cytosol have been identified with different forms of signalling that cause changes in the permeability of the outer mitochondrial membrane leading to the production of cytochrome C from the mitochondria and the formation of a multiple protein called the apoptosome (Borisenko, 2016).

2-10- Cell Viability

Cellular viability is the number of healthy cells in a sample. Measuring cellular viability is essential in all forms of cell culture. Cellular viability is calculated by dividing the number of live cells by the total number of cells (live and dead cells) (Madorran *et al*.,2024). Cell viability is expressed as a percentage of the control. Cell viability measurement is a necessary measure to analyse the efficacy of a new treatment and is often used in combination with cytotoxicity Assays to detect the effect of the treatment on cell viability because not all drugs and treatments work by the same mechanism (Mery *et al*.,2017).

2-11- Cytotoxicity

Cytotoxicity is a complex process that affects multiple metabolic pathways and indicators. After exposure to a toxic substance, cells will undergo either nicosis or apoptosis, which is accompanied by changes in nucleus shape, mitochondrial

function and cell permeability, resulting in loss of mitochondrial membrane strength and release of cytochrome C (Shamsee *et al*.,2019).

2-12- *NOX4* Gene

The *NOX4* gene is one of the seven members of the NOX family of enzymes that act as a catalytic subunit of Nicotinamide Adenine Dinucleotide (NADPH), an enzyme present in all cells that acts as an essential cofactor and substrate for a number of important cellular processes including participation in oxidative phosphorylation, ATP production, DNA repair, gene expression, intracellular calcium signalling, and immune functions, as well as an electron carrier that is used to temporarily store energy during cellular respiration. , an oxidising agent that accepts electrons from other molecules and becomes reduced and acts as an oxygen sensor that catalyses the reduction of molecular oxygen into different types of Reactive Oxygen Species (ROS) (Altenhöfer *et al*.,2012).

NADPH provides equivalents to reduce anabolic and redox reactions involved in protecting against ROS toxicity, and allowing the regeneration of GSH glutathione reductase. NADPH is also used for anabolic pathways, such as lipid synthesis, cholesterol synthesis, and fatty acid chain elongation. The NADPH system is also responsible for Generation of free radicals in immune cells and these radicals are used to destroy pathogens in a process called (respiratory burst) (Anilkumar *et al*.,2013).

The NADPH oxidase (NOX) family is widely recognised for its biological function of catalysing oxygen to produce reactive oxygen species (ROS). NADPH oxidase (NOX) has been identified as a multicomponent enzyme and one of the main sources of NOX. This family has so far comprised seven members (*NOX1, NOX2, NOX3, NOX4, NOX5, Duox1 and Duox2*), Their composition lead to the generation of hydrogen peroxide (H_2O_2) as the main product and sensor of oxygen *NOX4* -derived H_2O_2 plays diverse roles in cell proliferation, migration and apoptosis (Dewa *et al*.,2024; Goyal *et al*.,2005). These NOX isoforms differ in

terms of distribution in the organism, expression by cell type, location of subcellular structure, microproducts, and function and have been extensively investigated (Anilkumar *et al*.,2013).

NADPH (*NOX4*) was first identified by Shiose in 2001 (Mittal *et al*., 2007; Shiose *et al*.,2001), and *NOX4* is a human gene (Goyal *et al*,2005), which is the only form of the NOX family that produces hydrogen peroxide (H_2O_2). *NOX4* is widely distributed in human tissues and is particularly expressed in the kidney and blood vessels (Porporato *et al*.,2018; Block *et al*.,2009).

Increased generation reactive oxygen species (ROS) of Nicotinamide Adenine Dinucleotide (NADPH) derived promotes increased incidence of a variety of diseases such as chronic hepatitis, cardiovascular disease and cancer (Caramia *et al*.,2004). *NOX4* protects blood vessels from inflammatory stress (Geiszt *et al*., 2000), and modulation of NOX-dependent reactive oxygen species can lead to apoptosis in NOX4 -high-expressing cancer cells (Anwar *et al*.,2018). A study demonstrated that NOX4 facilitates some beneficial ROS-mediated adaptive responses (Sedeek *et al*.,2010).

The *NOX4* gene produces large amounts of hydrogen peroxide primarily, *NOX4* binds to the inner cell membrane protein and induces ROS generation making it the controller of cellular proliferation, differentiation, migration, apoptosis, senescence, fibrosis and angiogenesis. A study confirmed the high expression of the *NOX4* gene in many diseases such as cardiovascular diseases, including atherosclerosis, pulmonary fibrosis, high blood pressure, heart failure, and stroke (Lassegue & Griendling,2010).

Increased expression of the *NOX4* gene has been observed in colorectal cancer and is involved in metastasis, angiogenesis and apoptosis, expression of the *NOX4* gene in endothelial cells mediates endothelial activation, dysfunction, and cancer, which contributes to significantly altering cell proliferation, cell cycle, apoptosis,

migration and invasion in cell lines. The *NOX4* gene may have pathological importance and serve as a potential target for the treatment of cardiovascular diseases and cancer (De Santis *et al*.,2018 ; Goettsch *et al*.,2009).

2-13- *Bifidobacterium* spp.

Bifidobacterium was first isolated in 1899 by Henry Tisser, a French paediatrician at the Pasteur Institute in Paris, from the feces of breast-fed infants and named Bacillus bifides, which has a bacilliform morphology (Bertazzoni *et al*.,2001; Brock & Madigan,1991).

In 1907, Élie Metchnikoff, deputy director of the Pasteur Institute, theorised that lactic acid bacteria are beneficial to human health and observed that the good health and longevity of Bulgarian peasants was the result of their consumption of fermented milk products (Rasic, *et al*.,2003). It has also been suggested that oral ingestion of fermented bacterial cultures would cultivate beneficial bacteria in the inAssayinal tract European Probiotic Association- EPA,2012).

Several studies have reported a correlation between this bacterium and a decrease in diarrhoea in breastfed children and it has been used as a biological treatment for diarrhoea (Doré&Corthier,2010). The presence of *Bifidobacterium* in infant faeces has been observed to vary in abundance between breastfed and bottle-fed infants (Turroni *et al*.,2018). The incidence of diarrhoea caused by gastroinAssayinal infections in breastfed children is low and several studies have shown that this is due to the presence of Bifidobacterium bacteria in breast milk (Thibault *et al*.,2004; Chieric *et al*.,2003).

Studies confirmed that increasing *Bifidobacterium* bacterium reduces harmful bacteria (Yoshioka *et al*.,1983). It was observed that the pH value of breastfed babies' faeces is less than 6.0 while the pH value of formula-fed babies is more than 7.0 during the first seven weeks after birth due to the production of lactic acid

and acetic acid by the bacteria, which is why they are prescribed as food supplements in many studies (Saavedra & Tschernia,2002).

Bifidobacterium is an important microorganism as a natural flora, and the majority of its species are found in the large inAssayine, especially the colon, small inAssayine, breast milk, and the vagina (Ljungh & Wadstrom,2006). It constitutes approximately 99% of the commensal microbiota in the large inAssayine and 1% belongs to the rest of the commensal microbiota (Brankovic & Baras,2001).

The genus *Bifidobacterium* to date includes 48 different species (Tsuchida *et al*.,2014), isolated from various sources such as humans, animals, birds, insects, milk and dairy products, and honey (Ventura *et al*.,2014). All *Bifidobacterium* species are non-pathogenic except for one, *B. dentiuum*, which is isolated from dental caries (William *et al*.,2012) and is able to grow at temperatures up to 43C° (Zinedine & Faid,2007).

2-13-1-Classification and General Characteristics of *Bifidobacterium bifidum*

scientific name: *Bifidobacterium bifidum*is a bacterial species of the genus *B. bifidum* is one of the most common probiotic bacteria that can be found in the body of mammals, including humans (Sharma *et al*.,2021), and is classified according to Bergey's Manual of Systematic Bacteriology (Abdelhamid & El-Dougdoug,2021).

Kingdom : Procaryota

Phylum : Actinobacteria

Class : Actinobacteria

Order : Actinobacteriales

Family : Bifidobacteriaceae

Genus : *Bifidobacterium*

Species : *B. bifidum*

B. bifidum is a Gram-positive anaerobic bacterium that is neither motile nor spore-forming (Hoover *et al*.,2014). The bacteria are rod-shaped between short and long and may be branched and can be found living in groups, pairs or even independently, the colonies growing on the culture medium have convex and smooth edges with a characteristic odour (Palmer *et al*.,2007).

The sensitivity of *Bifidobacterium* genera to oxygen generally limits the activity of probiotics in anaerobic habitats (Bolduc *et al*.,2006). Recent research has reported that some strains of *Bifidobacterium* it shows a different growth nature for oxygen, low concentration of O_2 with the availability of CO_2 can have a stimulating effect on the growth of strains of *Bifidobacterium* depending on the nature of growth under different concentrations, of *Bifidobacterium* species have been classified into four sections, highly sensitive to O_2, sensitive to O_2, and insensitive to O_2, the primary factor responsible for the inhibition of aerobic growth is the production of hydrogen peroxide (H_2O_2) in the growth medium (Sonomoto *et al*.,2011).

It is negative for catalase and urease, negative for nitrate and indole, non-gas producing, acid and fermentation resistant (Braun,1981), does not produce NH_3 ammonia or H2S hydrogen sulfide from amino acids, growth temperature ranges from 37C° to 41C°, growth pH value ranges from 6.5 to 7.0, the proportion of guanine G and cytosine C in the DNA strand is 42-67% respectively (William,2012).

The genus *Bifidobacterium* possesses a unique fructose-6-phosphate phosphoketolase (F6PPK) pathway that is used for carbohydrate fermentation, it

is an enzyme of the genus *Bifidobacterium* and is responsible for the conversion of glucose to fructose-6-phosphate upon entering the cell (Abd *et al*.,2004).

The optimal medium for bacterial growth under anaerobic conditions is DeMan Rogosa sharp (MRS) medium and Trypticase phytone -Yeast (TPY) medium is a selective medium for it (Hadadji *et al*.,2005). It can also grow on fructose as a good source of carbon (Caescu *et al*.,2004).

Several studies have shown that *Bifidobacterium* is abundant in the gut of breastfed babies, especially in the first week after birth, but with age it may gradually decrease if not provided in the diet (Gronlunde *et al*.,2007). Their presence depends on several factors: food quality, use of antibiotics, fatigue and stress, *Bifidobacterium* species are naturally occurring beneficial bacteria in the gut and are probiotics that are used to replace the beneficial bacteria in the gut, which may have been lost after prolonged use of antibiotics (Santacruz *et al*., 2009), because *B. bifidum* is highly present in the large inAssayine, especially the colon, it competes with pathogens and yeasts for space and food (Servin, 2004), and is highly resistant to bile acids, which helps it to settle in the inAssayine permanently (Schell *et al*.,2002).

These bacteria have many methods that improve their biological effectiveness to reduce disease infections, including as an agent regulating the inAssayinal microbial balance and agents against pathogenic bacteria, that is, inhibiting pathogens and harmful bacteria that colonize or infect the inAssayinal mucosa due to their ability to produce organic acids, bacteriocins, and H_2O_2 , they have the ability to occupy bacterial and toxin binding sites on host epithelial cells as well as immunological effects by modulating systemic immune responses (Mack & Lebel,2003), It produces acetic acid and lactic acid ,they produce more acetic acid the ratio of acetic acid to lactic acid production is 2:3, respectively, and the effects of acetic acid are greater than those of lactic acid, especially against Gram-negative bacteria (Rasic & Korman,1983).

In a study indicating that *B. bifidum*, a natural microflora isolated from the inAssayines of breastfed children, could be given as a treatment against diarrhoea in children as an oral dose, this bacterial species has since been highlighted as a prevention or treatment against many diseases (Hamilton-Miller,2003).

Several in vitro and in vivo studies have suggested that B. bifidum may have the effect of improving the quantitative and qualitative profile of the inAssayinal microbiota (Zhang *et al*.,2015). The same results have been observed in clinical trials (Hatakka *et al*.,2008; Ohara *et al*.,2010), for example, regular consumption for 16 days was able to significantly increase diversity and microbiota in individuals with colorectal cancer (CRC) who had undergone colectomy, and in this case, the component of these inAssayinal microorganisms for these patients is akin to a natural therapy that has no side effects (Liu *et al*., 2011).

Another study confirmed the effectiveness of *B. bifidum* in digesting complex carbohydrates and produces various compounds which are important for gut health (Ju-hoon & Daniel,2010), namely short-chain fatty acids (SCFAs) such as glutamic acid and aspartic acid conjugated with linoleic acid (CLA), (Ladeira *et al*.,2023) and building some vitamins such as B2, B6, Riboflavin, Thiamin, Biotin, Purine, Pyrimidin and Vitamin K (Koboziev *et al*.,2013).

Studies have shown that *B. bifidum* bacteria combined with the drug Lactulose work on detoxification, in patients with hepatitis, it lowers the pH of the large inAssayine and restores the normal inAssayinal flora that has been reduced by liver diseases such as chronic liver cirrhosis by reducing the production of NH3 ammonia and free phenols and lowering serum cholesterol (Abt & Artis, 2009; Pinzone *et al*.,2012).

It regulates colon function, treating constipation in the elderly, lactose digestion, irritable bowel syndrome and ulcerative colitis caused by bacteria that necrotise the in Assayine , it is effective in reducing the side effects of ulcers

caused by *Helicobacter pylori* and contributes to the treatment of a skin disease called atopic eczema (Kumar *et al*.,2021).

Bifidobacterium is one of the original components of the large inAssayine in almost all ages, but with age, the proportion of this bacterium decreases significantly, especially in the elderly, and other bacterial species increase (Collado *et al*.,2008), many studies in this field have proven that cases of metabolic, immunological and various inAssayinal diseases coincide with the depletion of *Bifidobacterium* bacteria from the human gut microbiome, so many doctors recommend the use of these bacteria as probiotics either in capsules or pills to prevent possible diseases or as an alternative to the treatment of many diseases (Ladeira *et al*.,2023).

2-13-2- Probiotic Bacteria

The concept of probiotics came from the Greek word pro bios in 1908 by Nobel Prize-winning Russian researcher Ilya Metchnikoff, who observed a correlation between good health and the longevity of nomadic tribes inhabiting Bulgaria and Russia and their regular consumption of large quantities of yoghurt because contains lactic acid microbiomes that have the ability to survive in the gut and compete with pathogenic and unwanted gut microbiomes (Smug *et al*, 2014), the term probiotics was first used by Lilly & Still in 1965 as microorganisms that when consumed in appropriate amounts confer a health benefit to the host (Ladeira *et al*.,2023).

In 2013 the concept of probiotics was revisited by specialists International Scientific Association for Probiotics and Prebiotics (ISAPP), to discuss the field of probiotics, the Food and Agriculture Organisation/World Health Organization (FAO/WHO) probiotics definition was reinforced in 2001 as microorganisms with health and therapeutic benefits (Food and Agriculture Organization-FAO and World Health Organization-WHO,2002). There is also a treatment known as Probiotic Therapy (Colin *et al*.,2014), the aim of which is to increase the number

of beneficial microorganisms in the inAssayine and improve their effectiveness, thus restoring the natural microbial balance of the inAssayine. It is called Bacteriotherapy, which consists of three components: Prebiotics, Probiotics and Synbiotics (Guarner & Clin,2012).

The term Probiotics is associated with other terms related to human health benefits such as Direct Feed Microbial (DFM) which are types of beneficial bacteria and yeasts preserved under certain conditions and with specific numbers of microbiome dried and stored until use, Prebiotics which are fibre-rich foods that act as food for human microflora and are not affected by inAssayinal microorganisms that are not considered bio-enhancers such as *Escherichia coli*, and the term Synbiotics which is the combination of probiotics with natural prebiotics (Ladeira *et al*.,2023). Some studies suggest that a person who takes probiotics while taking antibiotics has a very high chance of not developing antibiotic-induced diarrhoea (Hempel *et al*.,2021).

Bacteria used as bioconjugates are non-pathogenic, non-toxic, and maintain their biological viability after growth in their culture media, during use, and throughout their preservation before consumption (Sumera *et al*.,2024), they are genetically stable, resistant to gastric acidity and bile salts, mostly are resistant to antibiotics and have a superior ability to adhere, colonise and reproduce in the inAssayinal lining and have good health effects for the host (Food and Agriculture Organization-FAO and World Health Organization-WHO,2002).

Scientific and clinical evidence, as well as the development of a number of effective probiotic products, has evolved and confirmed their importance in human health (Cosme *et al*.,2022), Studies have indicated that it has various effects on human health, such as lowering blood pressure, lowering cholesterol, preventing bowel diseases, improving the immune system (Bezirtzoglou & Stavropoulou,2011), and helping to overcome many diseases such as diarrhoea, constipation, obesity and depression (Elaine,2020).

Recently, the use of probiotics has witnessed great importance in treating many diseases, including oral and skin diseases, and cancer, especially colorectal cancer (Cornista *et al*.,2023), and they help in preventing many bacterial, fungal, and viral infections (Krenbiel *et al*.,2003).

Harvard University confirmed in 2021 that probiotics can do more than just improve gut health, they can also indirectly promote mental health. Research has also shown that the gut and the brain are interconnected via the so-called gut-brain axis, a connection between essential information between the brain and gut through the Vagus, which is the longest nerve in the human body (Grand, 2021).

Probiotics are used in many industrial fields such as food preservation, and certain types of microorganisms are added as bio-enhancers to many foods in the form of dried cells or fermented materials, giving them a desirable characteristic taste, increasing appetite and prolonging the stay of fats, nitrogen and calcium in the digestive tract (Guandalini *et al*.,2000).

Formula milk and fermented foods are a rich source of probiotic isolates and are known as good bacteria, microorganisms including bacteria, yeast and moulds are used as probiotics but the main microorganisms used as probiotics are bacteria of the genus *Lactobacillus rhamnosus*, *Lactococcus lactis*, *Bifidobacterium bifidum* and *Streptococcus salivarius*, (Ruben *et al*.,2023).

2-13-3- Biosurfactant (BS)

Biosurfactant are a diverse group of surface-active molecules with unique chemical properties that are of microbial origin and range in size from 500 to 1500 daltons (Marcelino *et al*.,2020). They were first discovered in the 1660s and have since been widely accepted as an effective alternative to their conventional Surfactant counterparts (Mukherjee *et al*.,2006).

Biosurfactant molecules are classically categorised as emulsifiers of hydrocarbon compounds and are widely applied in many fields including

pharmaceuticals, food processing, biodegradation, bioremediation, cosmetics and pest control management, they are substances formed on the surfaces of microorganisms or formed outside the living cell and secreted to the outside of cells to perform certain purposes for the cells and are amphiphilic containing hydrophilic molecules and other hydrophobic molecules and amphiph ilic , due to these properties, BS interacts with the lipid membranes of the cell and changes their physical and chemical nature and as a result, various biological functions of the target cells are lost. It reduces the surface tension of fluids, acts on interfaces or areas where surfaces meet and may change the nature of these surfaces from hydrophobic to hydrophilic, and lowers Surfactant Tension (SFT) and Interfacial Tension (IFT) (Marcelino *et al*.,2020).

Biosurfactant acts with similar mechanics to Surfactant and plays a key role in various processes such as emulsification, de-emulsification, wetting, forming, polymerisation and phasedispersion (Helmy *et al*.,2011), they are non-toxic and biodegradable (Banat *et al*.,2000).

Diverse microbial surfaces are selective in nature, environmentally friendly, characterised by long-term physico-chemical stability and can be mass-produced to withstand extreme environmental conditions and can be used in many applications (Rahman *et al*.,2002).

Biosurfactant (BS) and Bioemusifiers (BE) are synthesised from many microorganisms such as bacteria, fungi and yeasts and are used in many activities due to their dual familiarity and both possess emulsifying properties, the Biosurfactant contains one of the following in its structure: Glycolipide, Mycolic, lipoprotein, lipid composite-polysaccharide, lipopeptide, or phospholipide (Ramana & Karanth,1989).

Biosurfactant compounds of glycolipids and some short chains of lipopertide have low molecular weight, and polymeric compounds have high molecular

weight, and in both cases they have active surface properties that reduce surface tension (SFT) and interfacial tension (IFT) (Perfumo *et al*.,2009).

2-14- Biosurfactants Production

Biosurfactants are mainly produced by microorganisms bacteria, yeast and filamentous fungi, but can also be produced in animals (e.g. bile salts and phospholipids) and plants (e.g. saponin), (Aparna,2012)

Biosurfactants derived from microbes are known to have high emulsification capacity and lower surface tension, because the structure of Biosurfactants includes biomolecules such as lipids, proteins, and carbohydrates, they are more structurally complex than surfactants (Aparna,2012).

From an environmental perspective, Biosurfactants reduce environmental pollutants such as carbon dioxide (CO_2) and greenhouse gas emissions such as methane (CH_4) and nitrous oxide (N_2O), Microbes release Biosurfactants during the biodegradation of hydrocarbons, providing advantages, to maintain environmental sustainability, thus reducing reliance on chemical degradation methods (Rahman & Gakpe, 2008).

2-14-1- Types of Biosurfactants

Biosurfactants are categorised according to their chemical composition and microbiological origin and include monosaccharides, disaccharides, polysaccharides, anions or cations and are hydrophilic molecules of Biosurfactant (Kashif *et al*.,2022). In contrast, the hydrophobic fraction consists of saturated fatty acids and unsaturated fatty acids (Elsoud & Ahmed,2022).

Glycolipids are the most effective form of Biosurfactant consisting of fatty acids and carbohydrates connected to either an ester group or an ether group (Mnif *et al*.,2016).

The most common forms of Biosurfactant include Glycolipids, Lipopeptides , Polymeric, fatty acids and phospholipids (Ambechada,2024 ; Simões *et al*.,2024).

2-14-2- Advantages of Biosurfactants

Biosurfactants have many advantages compared to surfactants, including:

1- Biologically Degradable

Biosurfactants are suitable for biodegradation/bioabsorption because microorganisms break them down faster than their surfactant counterparts and chemical-based surfactants pose a risk to the environment (Femina *et. al*, 2021). As a result, naturally biodegradable Biosurfactants are seen as a more environmentally friendly, biocompatible and easily digestible alternative, making them useful in cosmetics, pharmaceuticals and therapeutic dietary supplements (Hogan *et al*.,2019; Sharma *et al*.,2021).

2- Low Toxicity

Biosurfactant is less dangerous than surfactant, and in addition, it is 50% more efficient than its surfactant counterpart (Yuan *et al*.,2014), it is therefore suitable for use in many applications such as pharmaceuticals, food and cosmetics, and a study using a Biosurfactant produced from *Bacillus subtilis* indicated that this compound is non-toxic and non-irritating, making it safer to use in detergent formulations (Fei *et al*.,2020).

Glycolipid and lipopeptide Biosurfactants generally have low toxicity, antimicrobial and skin surface moisturising properties and have therefore been considered a suitable material to replace surfactant in cosmetics and skin care products (Adu *et al*., 2020; Renuka, *et al*.,2023).

3- Specificity

Biosorbents are multifaceted macromolecules containing different functional groups, each responsible for a specific response quality, such as detoxification of certain pollutants, demulsification of commercial emulsions, and many medical and food applications (Femina *et al*.,2021).

4- Cost-Effective Raw Materials

Cheap natural resources can be used to synthesise Biosurfactants as their synthesis requires a high carbon content substrate, such as hydrocarbons, carbohydrates and lipids (Phetcharat *et al*.,2019). Their production is economically cheap and a large amount of Biosurfactants can be produced from waste and industrial residues, making them cheap compared to surfactants (Cassidy, 2001; Hospes *et al*., 2002).

2-14-3- Applications of Biosurfactants

Biosurfactants are important biotechnology products for industrial and medical applications due to their specific modes of action, low toxicity, relative ease of preparation and wide applicability, and are effective wetting and foaming agents, solvents, dispersants, dispersants, emulsifiers, and detergents such as soaps (Inès *et al*.,2023).

The properties of Biosurfactants can be widely used commercially (Banat *et al*.,2022). Global production of Biosurfactant volumes increased to about 17 million cubic tonnes by 2000 and economic research has confirmed the decreasing availability of chemical-based products on the market with increasing demand for biobased products (Olasanmi&Thring,2018), This has been identified by the expansion of biochemical patent rights (Tiso *et al*.,2017).

The increasing environmental awareness of environmental sustainability has also prompted increased interest in alternatives of biological origin that work to reduce environmental pollutants (Badmus *et al*.,2021).

Previous studies have confirmed that Biosurfactants have applications in many fields such as industrial as pesticides (Johnson *et al*.,2021) and medicine (Shekhar *et al*.,2015; Santos *et al*., 2016) which include:

1- Effective Anti-Cancer

Biosurfactants (BS) are amphiphilic compounds that exist as families of congeners and isomers that act as anti-tumour agents (Master & Markande,2023) it interferes with some cancer development processes, the antiproliferative activity of Bs makes them a potential anticancer agent, (BS) can arrest the cell cycle, induce apoptosis and inhibit cellular metastasis without any effect on healthy cells (Fracchia *et al*.,2012; Rodrigues,2011), one of the most important findings that has been reported is that surface biogenic dispersants have the ability to control cell differentiation, cell immune response, signal transduction, and control a variety of mammalian cell functions (Drakontis & Amin,2020).

Some microbial compounds or Biosurfactants such as glycolipids have the ability to provide differentiation and recognition of cancer cells and inhibit proliferation in humans (Rodrigues *et al*.,2006). A study showed that the Biosurfactant (Glycolipids) inhibits the growth, apoptosis, and differentiation of skin cancer cells (Zhao *et al*.,1999). Mannosylerythritol lipids have shown clear growth inhibition and differentiation activities against human leukaemia cells (Isoda & Nakahara,1997). Succinoyl trehalose inhibits the growth and induces the differentiation of the HL-60 cell line, a human leukaemia cell line that has been used in laboratory research on haematopoiesis and physiology (Sudo *et al*., 2000), and the human basophil leukemia cell line KU812 (Blom *et al*.,1992).

In addition, lipopeptides have been extensively studied for their potential anti-tumour activities many researchers have confirmed the efficacy of Biosurfactants and other lipopeptides against different cancer cell lines (Liu *et al*.,2010; Seydlová & Svobodová, 2008; Sivapathasekaran *et al*.,2010).

evaluated the effect of Biosurfactants on the human colon cancer cell line LoVo and showed that lipopeptide possesses potent growth inhibitory activity by inducing apoptosis and cell cycle arrest (Kim *et al*.,2007).

While Biosurfactants are able to disrupt cell membranes by degrading and increasing membrane permeability (Gudiña *et al*.,2013).

Research by (Cao *et al*.,2010) showed that Biosurfactant causes apoptosis through the mitochondrial/caspas pathway (ROS)/reactive oxygen species/(JNK) Jun N-terminal mediated pathway, where mitochondrial loses its function by caspase proteases and generation of reactive oxygen species during apoptosis , the same researchers also demonstrated the toxic effect of the Biosurfactants against K562 human chronic myeloid leukaemia cells and BEL7402 hepatocellular carcinoma cells.

evaluated the effect of lipopeptides produced by *Bacillus subtilis* on Bcap-37 breast cancer cell lines and demonstrated that these compounds induce apoptosis in a dose-dependent manner (Liu *et al*.,2010), that Biosurfactants inhibited the growth of MCF7 human breast cancer cells in a dose-dependent manner as well (Lee *et al*.,2012), this is why various glycolipids are used to detect and treat cancer and thus have an effective role against cancerous tumors in humans (Krishnaswamy *et al*.,2008).

A novel cytotoxic activity against cancer cell lines was revealed by lipopeptides, from Biosurfactants and fengycins produced by *Bacillus circulans* DMS2, a marine microorganism, as using lipopeptides in a dose-dependent manner as an antiproliferative agent after 24 hours of treatment, more than 90% inhibition of proliferation on human HCT 15 or HT 29 colon cancer cell lines could be achieved by concentrating purified lipopeptides at 300 µg/ml (Sivapathasekaran *et al*.,2010).

The inhibitory performance of Biosurfactants was also evaluated after conducting several studies to determine the effects against cancer cell lines when compared to non-cancerous cell lines , *Serratamolide* AT514 found in a group of cyclic depsipeptides produced by the bacterium *Serratia marcescens*, was

discovered, as a practical inducer of apoptosis in several cell lines in β-chronic lymphocytic leukaemia cells, in various human tumors, this mainly involves the mitochondria-mediated apoptosis pathway(Matsuyama *et al*.,2010), Biosurfactant research against the human lymphoma cell line AT514 is progressing towards clinical applications in medical oncology (Fechtner *et al*., 2017).

2- Effective Anti-Microbial

Biosurfactant are Antibacterial, Anti-viral, Anti-fungal, Anti-tumour, Anti-mycoplasma and Anti-inflammatory (Seydlova&Svobodova,2008), Biosurfactant of the lipopeptide type have activity against various cell membranes, whether Gram-positive or Gram-negative bacteria. Lipopeptide affects the integrity of the membrane but not important cellular processes, many antibiotics can be made from Biosurfactant (Rodrigues & Teixeira,2010).

Studies have indicated that the Biosurfactant derived from *Bacillus circulans* has antimicrobial activity as well as against several Gram-positive and Gram-negative pathogenic bacteria, *Micrococcus flavus*, *Bacillus pumilus*, *Mycobacterium smegmatis*, *Escherichia coli*, *Serratia marcescens* and *Proteus vulgaris* are among the organisms that produce effective Biosurfactant (Das *et al*.,2008), the chemical composition of the Biosurfactant of these species has overlapping patterns such as lipopeptides and lichenysin, it has been observed to have an antagonistic effect against Methicillin-resistant Staphylococcus aureus (MRSA) and other multi-drug-resistant (MDR) pathogenic strains (Cameotra *et al*.,2004). this confirms that Biosurfactant can be used as drugs in antimicrobial chemotherapy due to its nature (Fracchia *et al*.,2012).

3- Effective Anti-Adhesive

Biosurfactant have anti-adhesion properties to solid surfaces or infected biological surfaces. Bacteria can colonize medical devices and food processing surfaces, changing their properties. Moreover, they may represent an important

source of contamination, as pathogenic bacteria produce biofilms, which are more resistant to antimicrobial agents and disinfectants (Palmer & White,1997).

Adhesion of bacteria to surfaces is one of the initial steps leading to the formation of biofilms (Donlan & Costeron,2002), thus, actions that prevent or reduce bacterial attachment may reduce biofilm formation and the presence of polymers, protein and other molecules in the medium can affect bacterial attachment because these particles can accumulate on surfaces and thus change their properties , factors affecting this include hydrophobicity, electrostatic charges, and surface free energies (Goulter *et al*., 2009), Much effort has been directed to avoid bacterial adhesion, studies have been conducted on changing the surface properties of contact surfaces by conditioning (BS) (Meylheuc *et al*.,2001; Nitschke *et al*.,2009).

(BS) of microbial origin has many advantages over synthetic surfactants BS has low toxicity, is biodegradable, offers chemical diversity, is effective under extreme environmental conditions - such as temperature, pH, and ionic strength - exhibits strong surface activity, emulsifiability, and has antimicrobial and anti-adhesive properties (Singh&Cameotra, 2004; Gautam &Tyagi,2006; Nitschke *et al*.,2007).

Although the anti-adhesive mechanisms of Biosurfactant are not fully understood, they have anti-adhesive properties against many microorganisms (Rodrigues *et al*.,2011), Showed that vinyl coating before urethral catheterisation by activating a surfactin solution caused a significant reduction in biofilm production by *Salamonella typhimurium*, *S.enterica*, *Escherichia coli* and *Proteus mirabilis* (Rodrigues *et al*.,2004).

Research has also that Biosurfactant isolated from Lactic acid bacteria has shown anti-adhesive activity, but its antimicrobial activity has only been characterised for a few strains (Gudin *et al*.,2010).

(BS) has been used against microorganisms responsible for diseases and infections in the urinary and vaginal tract, gastroinAssayinal tract, and skin, as well as against cancer cell lines, making it a suitable alternative to conventional antibiotics (Ibrahim *et al*.,2019; Sajid *et al*.,2020; Ceresa *et al*.,2023).

Chapter Three

Materials and Methods

3- Materials and Methods

3-1- Materials

3-1-1- Tools Used in the Study

A number of tools were used, shown in Table (3-1).

Table (3-1): Laboratory Tools and Origin

No.	Name of Tools	Company (Country)
1-	Conical flasks	BBL (USA)
2-	Cooled Box	Syria
3-	Durham Tubes	Shndon (England)
4-	Eppendorfs Tubs	Qrenier (Germany)
5-	Filter Paper	China
6-	Flask	BBL (USA)
7-	Loop	Loop Rhndon (England)
8-	Membrane Filter	Difico (USA)
9-	Micropipettes	Germany
10-	Microtiter Plate (U shape)	Biohit (Finland)
11-	Paraflim	BDH (England)
12-	Petri Dishes	Plastilab (Lebanon)
13-	Pipets Tips	China
14-	Slides and Coverslips	Meheco (China)
15-	Syringes	Mecheco (China)
16-	Tubes	Afco-Dispo (Jorden)

3-1-2- Laboratory Device Used in the Study

A number of devices were used in the current study as shown in Table (3-2).

Table (3-2): Laboratory Equipment and Origin

No.	Name of Device	Company (Country)
1-	Autoclave	Labtech (Korea)
2-	Benzen Burner	Shndon (England)
3-	Centrifuge	Hettich (Germany)
4-	Computer	Lenovo (USA)

5-	Cooling Centrifuge	Hettich (Germany)
6-	Deionized Water	Ogawa (Japan)
7-	Distiller	Ogawa (Japan)
8-	Electrical Sensitive Balance	Satorius (Germany)
9-	Electrophoresis	Biotek (USA)
10-	ELISA	Biotek (USA)
11-	Flow Cytometry	BD Bioscience (USA)
12-	High Content Screening Assay Scan (HCS)	Thermos Scientific (USA)
13-	Incubator	Binder (Germany)
14-	Inverted Microscope	OLYMPUS (Japan)
15-	Laminar Flow Hood	K&K Scientific Supplier (Korea)
16-	Lyophilizer	Memmert (Germany)
17-	Magnetic Stirrer	Scotech (Germany)
18-	Microscope	OLYMPUS (Japan)
19-	Microscope Camera	Olympus (Japan)
20-	Microtiter Reader	Gennex lab (USA)
21-	Oven	Memmert (Germany)
22-	Polymerase Chain Reaction	Biotek (USA)
23-	NanoDrop Spectrophotometer	Biotek (USA)
24-	Refrigerator	LG (korea)
25-	Rotary Evaporator	(II, Slovenia)
26-	Shaker Incubator	Labtech (Korea)
27-	Vitek 2 System	BioMerieux (France)
28-	vortex Mixer	Biotek (USA)
29-	Water Bath	Memmert (Germany)

3-1-3- Biological and Chemical Materials Used in the Study

A number of materials were used in the current study as shown in Table (3-3)

Table (3-3): Biological and Chemical Substances and Origin

No.	Name of Materials	Company (Country)
1-	Absolute Ethanol	BDH (England)
2-	Ager – Agar	Oxoid (France)
3-	Alpha Naphthol	BDH (England)
4-	Ascorbic Acid	Oxoid (England)

5-	$BaCl_2$	BDH (England)
6-	$BaCl_2H_2O$	BDH chemical ltd (England)
7-	Beef Extract	Oxoid (England)
8-	C6H5-CH3	BDH (England)
9-	cell lines: Human Rectal Tumor-18 [HRT-18]	European Collection of Authenticated Cell Cultures (United Kingdom)
10-	cell lines: Human Dermal Fibroblasts – neonatal HDFn	Thermo Fisher Scientific (Gibco™) (USA)
11-	CH_3COOH	BDH Chemical Ltd (England)
12-	Coconut Oil	Iraq
13-	$CuSO_4$	BDH Chemical Ltd (England)
14-	Dimethyl Sulfoxide (DMSO)	Oxoid (England)
15-	Ethanol	Fluka (Switzerland)
16-	Ethylene Diamine Tetra (EDTA) Acetic acid	Capricorn (Germany)
17-	Fetal Bovine Serum (FBS)	Capricorn (Germany)
18-	Free Nuclease Water	Bioneer (Korea)
19-	Fresh Eggs	Local Markets in Holy Kerbala
20-	Gelatin	Oxoid (England)
21-	Gingely Oil	Iraq
22-	Glycreol	BDH (England)
23-	Gram Stains	UK BDH
24-	H_2O_2	BDH Chemical Ltd (England)
25-	H2SO4	BDH(Englaand)
26-	HCL	BDH Chemical Ltd (England)
27-	HCS Assay Kit	Thermos Scientific (USA)
28-	Iodine	Sigma (USA)
29-	Kerosene	BDH (England)
30-	KI	BDH Chemical Ltd (England)
31-	KNO_3	BDH Chemical Ltd (England)
32-	KOH	Thomas Baker
33-	Kovac's reagent	Oxoid (England)
34-	L-arginine Monohydrochloride	Oxoid
35-	L-Cystiene-HCL	Oxoid
36-	Lugol's Solution	Oxoid
37-	Malachite Green	HI-media (India)
38-	Methanol	B.D.H (U.K)
39-	MTT Assay kit (stain)	OZ BIOSCIENCES (France)

40-	NaCl	BDH Chemical Ltd (England)
41-	$NaHCO_3$	Sigma (USA)
42-	NaOH	Fluka (Switzerland)
43-	Nessler Reagent	Oxoid (England)
44-	NH_4Cl	BDH Chemical Ltd (England
45-	Normal Saline	BDH (England)
46-	Peptone Water	HI-Media India
47-	Phenol Red	HI-Media India
48-	pH-Meter	Memmert (Germany)
49-	Povidone Iodine 10%	Iraq
50-	Sulfanilic Acid	BDH Chemical Ltd (England)
51-	Trypsin Solution	Sigma (USA)
52-	Trypsin/ EDTA	Capricorn (Germany)
53-	Urea	Oxoid (England)
54-	α-Napthalamine	BDH chemical ltd (England)

3-1-4- Sugars Used in the Study

A number of sugars were used in the current study as shown in Table (3-4)

Table (3-4): Sugars Used and Origin

N0.	Name of Sugars	Company (Country)
1-	Arabinose	Merck (Germany)
2-	Fructose	Merck
3-	Galatose	Merck
4-	Glucose	B.D.H (England)
5-	Lactose	Merck
6-	Maltose	Merck
7-	Mannitol	Merck
8-	Mannose	Merck
9-	Raffinose	Merck
10-	Sucrose	UK BDH
11-	Xylose	Merck

3-1-5- The Culture Media and the Purpose of their Use

In the current study, a number of culture media were used, which were prepared according to the manufacturer's instructions and shown in Table (3-5).

Table (3-5): Cultivation Media, Purpose of Use, and Origin

No.	Culture media	Purpose of their Use	Company (Country)
1-	(MRS) (De Man Rogosa Sharp Agar)	For the Purpose of Growing, Purifying and Revitalizing *Bifidobacterium bifidum* Bacteria and Preserving them on the Inclined Medium	Oxoid (England)
2-	(MRS) Broth De Man Rogosa Sharp Agar	For the Purpose of Growing and Revitalizing *Bifidobacterium bifidum* Bacteria and Preserving them with the Use of Glycerol	Oxoid
3-	Agar-Agar	To Diagnose the Mobility of Bacteria	Prepared in the laboratory
4-	Blood Agar Base	Enriched Medium for Growing All Types of Bacteria	Oxoid
5-	Motility medium	To Conduct a Motility Assay and Investigate whe ther or not the Bacteria Can Move	Oxoid
6-	Nutrient Agar Media	To Cultivate and Activate Pure Bacterial Isolates	Himedia (India)

7-	Nutrient Broth	To Develop Bacterial Isolates and Activate them to Conduct Biochemical Assays and Prepare Various Bacterial Plankton, Storing the Samples for a Period of Time at a Temperature of -20C°	Himedia
8-	Nutrient Gelatin	To Conduct a Gelatin Liquefaction Assay and to Investigate the Ability of Bacteria to Liquefy Gelatin	Biosciences (France)
9-	Pepton Water Medium	To Investigate the Ability of Bacteria to Degrade Tryptophan and Produce Indole	Himedia (India)
10-	RPMI-1640	To Culture media for the growth of normal and cancer cell lines (Activate of the Cells line)	Capricorn (Germany)
11-	Simmon Citrate	To Conduct a Jacket Consumption Assay	Himedia (India)
12-	Urea Agar	To Conduct a Urease Assay and to Investigate the Potential of Bacteria to Produce the Enzyme Urease	OZ Biosciences

3-1-6- Study Design

The axes to carry out the study shown in fismee below (3-1).

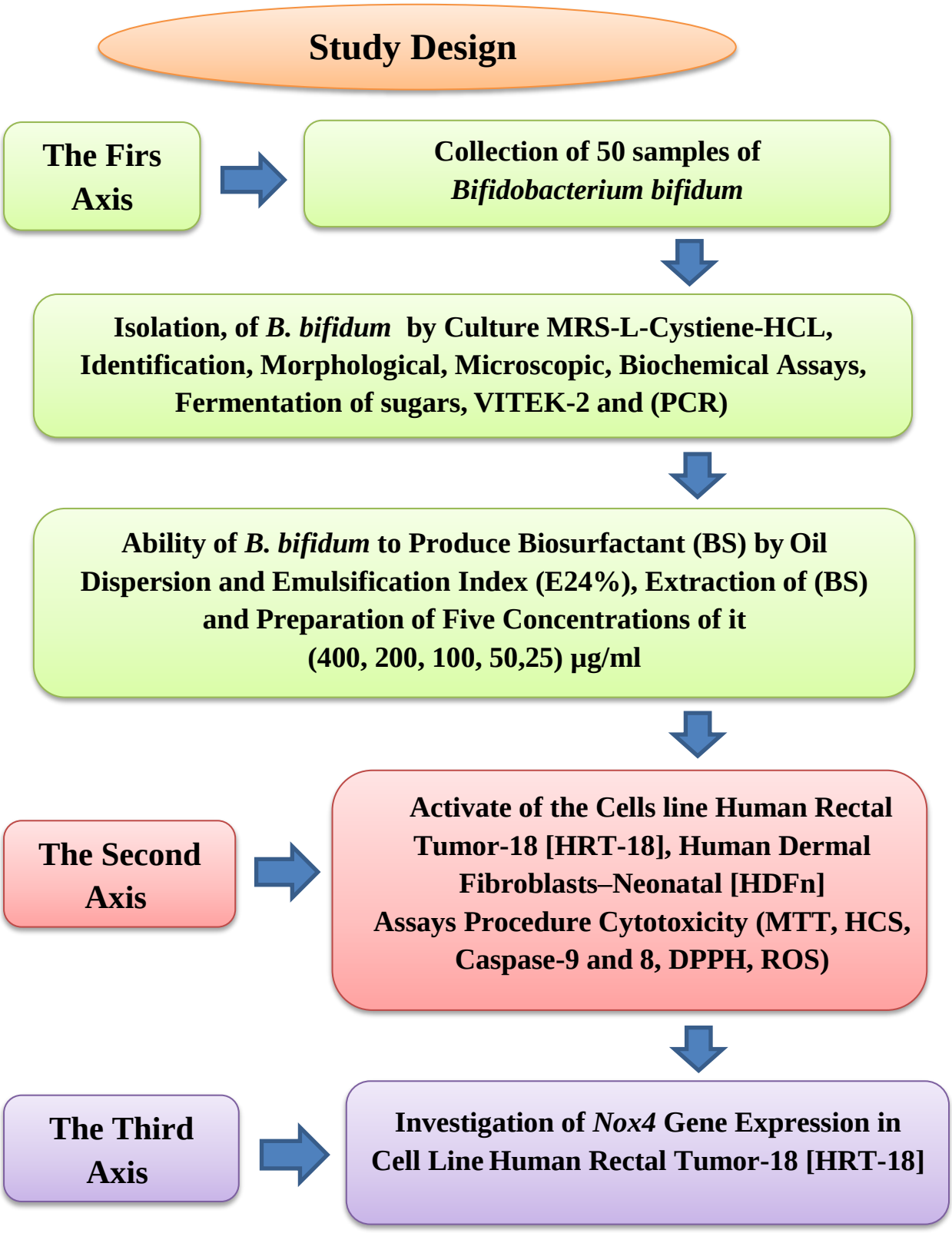


Figure (3-1) Study Axes

3-2- Methods

3-2-1- Sample Collection

Samples were collected from April 2024 to the end of September 2024 from fresh cow's milk, lactating mothers' milk, and both imported and locally produced dairy products such as yogurt, which are known potential sources of *Bifidobacterium bifidum*, fifty samples were included, and five isolates of *B. bifidum* were obtained: were obtained: two from dairy products, two from cow's milk, and one from human milk. The identity of the isolates was confirmed through microscopic and laboratory examinations, using the VITEK-2 Compact system and Polymerase Chain Reaction (PCR) technique.

3-2-2- Preparation of Culture Media

The culture media required in the current study were prepared as follows:

3-2-2-1- Ready-Made Culture Media

These media were prepared according to the manufacturers' instructions and sterilised by autoclaving at 121C°and 15 lb/in². After pouring, they were incubated in the incubator for 24 hours to ensure that they were not contaminated, then stored in a refrigerator at 4C° until use.

3-2-2-2- Prepared Culture Media

These media were prepared as follows:

3-2-2-2-1- MRS-L-Cystiene

a) Solution L-Cytiene-HCL

The solution was prepared by dissolving 5 g of L-Cystiene-HCl in distilled water, and after mixing it, the volume was increased to 100 ml of distilled water and filtered for sterilization with fine filters diameter of 0.22 microliters.

b) Middle MRS-L-Cystiene

MRS was prepared according to the Manufacturer company's instructions, then complete the volume to 900 ml of distilled water. It was sterilized in an autoclave and then left to cool to 50C°, then add 100 ml of sterile L-Cystiene-HCL solution

to it and mix well, then pour it into sterile Petri dishes. This medium was prepared for the purpose of growing and isolating *B. bifidum* (Atlas,2010).

3-2-2-2-2- Blood Agar Base

The blood agar medium was prepared by adding 5% of human blood to autoclaved blood agar and allowed to cool to 45-50C° according to the manufacturer's instructions and then poured into sterile Petri dishes. This medium was used to grow and isolate bacteria for the KOH Assay, which confirms the result of the Gram stain Assay. (Medical Lab Notes,2022).

3-2-2-2-3- Gelatin Medium

Prepare gelatin medium by adding 120g of gelatin to 1 liter of Nutrient broth, sterilize in an autoclave and distribute it into tubes at an amount of 5 ml for each tube, This medium was used to investigate the potential of bacteria in producing the enzyme gelatinase (Logan & Vosa,2009).

3-2-2-2-4- Starch Medium

The starch medium was prepared by adding starch to Nutrient agar medium at a rate of 2%, sterilizing it in an autoclave, and pouring it into Petri dishes in an amount of 25 ml in each dish. This medium was used to determine the ability of bacteria to degrade starch (Atlas *et al*.,1993).

3-2-2-2-5- Arginine – MRS Broth

Thy was prepared using MRS broth with the addition of 0.3% w/v L-arginine monohydrochloride and sterilized using an autoclave (Harrigans & MacCance,1976).

3-2-2-2-6- Nitrate Reduction Broth

Thy was prepared by dissolving 3g of Meat Exteact, 1g of KNO_3, and 5g of Peptone in a liter of distilled water, adjusting the pH to 7, then pouring the medium into tubes with an amount of 5ml for each tube, and then it was sterilized with an autoclave. This medium was used to investigate the potential of bacteria in producing the Nitrate Reductase. Which converts nitrate (NO_3) to nitrite (NO_2) (Logan & Vosa,2009).

3-2-2-2-7- Milk Agar Skim

Thy was prepared by adding 12.5 ml of Milk Skim to 87.5 ml of Nutrient agar prepared according to the manufacturer's instructions. It was sterilized with an autoclave and left to reach a temperature of 45-50C°. Then it was poured into sterilized Petri dishes. This medium was used to investigate the ability of bacteria to produce Protease, which works on casein analysis (Collee *et al*., 1996).

3-2-2-2-8- Egg-Yolk-Agar

Thy was prepared by adding egg yolk to 15% Nutrient agar sterilized at a temperature of 50-55C°. This medium was used to investigate the potential of bacteria in producing Lecithinase and Lipase (Cruickshank *et al*.,1975).

3-2-2-2-9- Carbohydrate Fermentation Medium

Thy was prepared by dissolving 10 g of Peptone, 10 g of Meat Exteact, 5 g of NaCl, and 0.08 of Phenol red in 1 liter of distilled water, adjusting the pH to 7.4, and pouring the medium into glass tubes with an amount of 5 ml for each tube, which was sterilized in an autoclave, and the medium was left to cool, and sterilized sugar was added to it by filtration. by 1% (MacFaddin, 2000).

3-2-2-2-10- Motility Medium

This medium was prepared from 100 ml of Nutrient broth according to the manufacturer's instructions, and 0.4 g of agar-agar was added to it. It was sterilized in an outoclave and distributed in plastic tubes at an amount of 5 ml for each tube. The medium was left to solidify in an Portrait mode. This medium was used to investigate the ability of bacteria to move (Forbes *et al*.,2007).

3-2-3- Preparing the Solutions and Reagents Used

The solutions and reagents used in the study were prepared as follows:

3-2-3-1- Macfarland Standard Solution

a) Barium Chloride Solution $BaCl_2$.

It was prepared by dissolving 1.175 g of aqueous barium chloride in 100 ml of distilled water (Atlas *et al*.,1995).

b) Concentrated Sulfuric Acid Solution H2SO4 at a Concentration of 1%.

It was Prepared by adding 1ml of H2SO4 to 99ml of distilled water, then adding 0.5ml of solution a to 99.5ml of solution b, mixing the solution well, dispensing 5-4ml into glass tubes with a tight cap to prevent evaporation and kept in the dark at room temperature until use. This solution is intended as a standard solution for titration of bacterial cell counts and to obtain a solution of 1.5×108 cells/ml, prepared according to the manufacturers' instructions.

3-2-3-2- Normal Saline

The physiological saline solution (NS) was prepared) 0.85 g of NaCl was dissolved in 100 ml of distilled water, sterilised with autoclave and stored at 4C° until use. This solution was used to prepare the dilutions required in this study (Atlas *et al*.,1995).

3-2-3-3- Phosphate – Buffer Saline (PBS)

This solution was prepared according to the manufacturer's instructions, then sterilised with autoclave and kept in a refrigerator at 4C° until use.

3-2-3-4- Malachite Green

It was prepared by dissolving 0.5g of Malachite green in a volume of distilled water and after dissolving the volume was completed to 100ml with distilled water. This stain has been used to investigate the spore-forming potential of purified bacterial isolates (Pommerville,2007).

3-2-3-5- KOH Solution

It was prepared by dissolving 3 g KOH in 100 ml of distilled water (Atlas, 2010).

3-2-3-6- Oxidase Reagent

The reagent was prepared instantaneously by dissolving 1g of (Tertramethyl P-phenylen-diamine dihydro chloride) in 100 ml distilled water to obtain a violet-coloured liquid that was placed in a dark, sterile vial (Tang & Stratton, 2006).

3-2-3-7- Catalase Reagent

H_2O_2 at 3% concentration was used to investigate the ability of bacteria to produce catalase (Tang & Stratton, 2006).

3-2-3-8- Kovac's Reagent

Use the manufacturer's ready-made reagent.

3-2-3-9- Ureae Solution

Prepared by dissolving 20 g of urea in 100 ml of distilled water and filtered through 0.22 Membrane filters (Macfaddin,2000).

3-2-3-10- Solutions Used to Adjust PH

a) 0.5 N HCl solution

b) 0.5 N NaOH solution

3-2-3-11- Nitrate Reduction Reagent Solutions

a) Sulfanilic acid solution was prepared by dissolving 0.8 g of sulfanilic acid in 100 ml of acetic acid of 5 mol .

b) The solution of α-naphthylamine was prepared by dissolving 0.5g of naphthylamine in 100 ml of 5 mol% acetic acid, and the nitrate reduction reagent was prepared anionically from a mixture of two equal parts of solutions A and B (Macfaddin,2000).

3-2-3-12- Sugar Solutions

It was prepared by dissolving 1 g of each of the following types of sugars (Arabinose, Fructose, Galatose, Glucose, Lactose, Maltose, Mannitol, Mannose, Raffinose, Sucrose, Xylose) each type separately in 100 ml of distilled water and sterilizing the solutions by filtration using membrane filters. Millipore filter 0.22 microliter (Macfaddin,2000).

Xylose

3-2-4- Sample Culturing

After collecting the samples, they were transferred to the laboratory and grown by taking a portion of the products with a sterile conveyor and directly cultured on Petri dishes containing MRS-L-Cytiene culture medium prepared according to (3-2-2-2-1) and incubated anaerobically using an anaerobic jar with a gas generating kit (gas pak) and kept in the incubator at 37C° for 48 h. the incubator was incubated at 37C° for 48 h.

Samples were grown in two ways:

1) A small portion of milk or one of the collected products was taken with a sterile carrier and directly seeded onto the MRS-L-Cystiene solid medium in a planned manner without any dilution and the plates were incubated under anaerobic conditions using Anaerobic Jar with a gas pak in the incubator at 37C° for 48 hours.

2) Milk or one of the collected products was diluted with 0.1% peptone water prepared and sterilised according to the manufacturer's instructions, then a small portion of the suspension was taken with a sterile vector and cultivation onto MRS-L-Cystiene solid media in the same manner as described in (1) (Rocío *et al*.,2009).

3-2-5- Sample Diagnosis

After obtaining single colonies from positive cultures, *B. bifidum* was diagnosed according to Bergey's Manual of Systematic Bacteriology,2015 (Whitman,2015).

3-2-5-1- Morphological Characteristics

The phenotypic characteristics of the bacterial colonies growing on MRS-L-Cystiene medium were determined by observing the shape, colour, texture and odour of the growing colonies.

3-2-5-2- Microscopic Examinations

The laboratory Assays consisted of transferring a part of the bacterial culture grown on MRS-L-Cystiene solid medium to a slide by a sterile Loop and after fixing and staining with Gram Stain, and after the slide dried, it was examined under the oil lens of a light microscope to observe the shape of the bacterial cells and the result of Gram staining.

3-2-6- Biochemical Assay

Biochemical Assays included the following:

3-2-6-1- KOH Assay

The KOH Assay is a confirmatory Assay of the Gram Stain Assay result by taking a single colony growing on the basic blood medium prepared according to paragraph (3-2-2-2-2) and mixed with an amount of KOH 3% prepared according to paragraph (3-2-3-5). Loop or stick to see the nature of the substance formed. If

the bacteria are Gram-positive, the gel is not formed, while if the bacteria are Gram-negative, the gel is formed within 60 seconds (Markey *et al*., 2014).

3-2-6-2- Oxidase Assay

This Assay was carried out by placing drops of oxidase reagent prepared in paragraph (3-2-3-6), where it was transfer by sterile stick a pure colony to a clean and dry filter paper on which drops of oxidase reagent were immediately placed. If the colour of the colony turned dark purple immediately, it is evidence of a positive Assay and the ability of the bacteria to produce oxidase enzyme (MacFaddin,2000).

3-2-6-3- Catalase Assay

A portion of the top of the bacterial growth colonies was taken from the MRS-L-Cystiene solid medium and placed on a clean glass slide and a drop of H2O2 reagent prepared according to paragraph (3-2-3-7) was added. The appearance of bubbles indicates a positive Assay and the ability of the bacteria to produce catalase enzyme (MacFaddin,2000). This Assay is used to investigate the potential of bacteria to produce catalase enzyme.

3-2-6-4- Indol Assay

This Assay was represented by inoculating the tubes containing the peptone water medium with bacterial colonies and then incubating the tubes at 37C° for 24 to 48 hours after the end of the incubation period, we observe the presence of turbidity, evidence of bacterial growth. Two drops of Kovac′s reagent prepared according to paragraph (3-2-3-8) were added according to the manufacturer's instructions in case a red ring is formed, indicating a positive Assay and showing the ability of the bacteria to analyse the amino acid tryptophan and produce indole Macfaddin (MacFaddin,2000).

3-2-6-5- Citrate Utilization Assay

This Assay was carried out by inoculating tubes containing Simmon Citrate slant medium prepared according to the manufacturer's instructions by stabbing and planning with bacteria and incubating at 37C° for 24 to 48 hours. The color of the medium changed from green to blue as evidence of the Assay's positivity and the ability of the isolates to consume citrate as the sole carbon source (MacFaddin, 2000).

3-2-6-6- Urease Assay

Tubes with slant surfaces containing urea agar medium prepared according to the manufacturer's instructions were inoculated with bacteria by stabbing and planning method and then incubated at 37C° for 24 to 48 h. The Assay result was read by turning the pale yellow colour of the medium to pink, indicating a positive Assay, as the bacterial colonies can produce Urease and degrade Urea into NH3 and CO_2 (Collins *et al*., 2004).

3-2-6-7- Gelatin liquefaction Assay

The Assay was carried out by inoculating tubes containing gelatin medium prepared according to (3-2-2-2-3) with bacteria by the stabbing method using a needle in the centre of the medium and then incubating the tubes at 37C° for 24 to 48 h. If the gelatin does not harden after incubation at 4C° for 10 min, this indicates a positive result (MacFaddin,2000).

3-2-6-8- Starch Hydrolysis Assay

This Assay was carried out by inoculating the solid Starch medium prepared according to (3-2-2-2-4) with a portion of the 18-24 h old bacterial culture in a Streak Plate Method and incubating the plates at 37C°. After the end of the incubation period, Lugol's solution was added to the growing bacterial culture on the culture medium and left for 1 to 2 min. The Assay result was read as a clear or blue bright area around the colonies (MacFaddin,2000).

3-2-6-9- Ammonia Production Assay from Arginine

This Assay consisted of inoculating the liquid MRS-arginine medium prepared according to paragraph (3-2-2-2-5) with part of the 24-hour-old bacterial culture and then incubated at 37C° for 7 days. After the end of the incubation period, 1ml of the inoculated medium was taken and placed in a Assay tube and 1ml of Nessler's reagent was added to it and the Assay result was read by changing the colour of the medium to red immediately after adding the reagent (Harrigan,1998).

3-2-6-10- Nitrate Reduction Assay

This Assay consisted of inoculating the nitrate reduction medium prepared according to (3-2-2-2-6) with a portion of the 18-24 hour-old bacterial culture and then adding 0.1 ml of nitrate reduction reagent prepared according to (3-2-3-11) to the tube and reading the result according to the colour changes of the medium (MacFaddin,2000).

3-2-6-11- Casein Hydrolysis Assay

This Assay consisted of inoculating the milk agar medium prepared according to paragraph (3-2-2-2-7) with 24-hour-old bacterial colonies and then incubated at 37C° for 4-7 days. If clear areas appear around it the growing bacterial colonies, this indicates the degradation of milk proteins (casein) and the result is positive; if the medium remains cloudy, this indicates that casein is not degraded and the Assay result is negative (Koneman *et al*.,1997).

3-2-6-12- Detection of the Enzyme Lecithinase and Lipase

This Assay was carried out by inoculating the egg yolk medium prepared according to paragraph (3-2-2-2-8) with part of the bacterial culture and incubating at 37C° for a period of 24 to 48 hours. If clear areas form around the growing bacterial colonies, this indicates effectiveness of the lecithinase can also be checked for the lipase in the same Assay by covering the medium with a generous amount of copper sulphate solution (CuSO4) for 20 minutes. Then the

excess solution was removed and the dish was dried by placing it in the incubator for a short period of time. The Assay result was read by the appearance of a greenish blue color in the sites of fat decomposition. This a evidence of the effectiveness of the lipase enzyme in decomposing fats (Collee *et al*.,1996; Cruickshank *et al*.,1975).

3-2-6-13- Motility Assay

Tubes containing the motility medium prepared according to paragraph (3-2-2-2-10) were inoculated with bacteria by the stabbing method and then incubated at 37C° for 24 to 48 hours in a vertical position. This assay was used to detect the motility of the bacteria as the lack of bacterial growth beyond the borders of the stabbing indicates that the bacteria cannot move (Collins *et al*.,2004).

3-2-6-14- Growth Assay at Different Temperatures

This Assay consisted of using MRS-broth medium prepared according to (3-2-2-2-1) distributed in sterile Assay tubes that were inoculated with bacterial cultures and then incubated anaerobically using a gas generating kit (gas pak) and placed in a Shaker Incubator for 10 days at different temperatures from 5 to 45C°. The potential of the bacteria to grow at these different temperatures was compared by comparing the density of growth in the form of turbidity or sedimentation (Buck *et al*.,1995).

3-2-6-15- Detection of Gas Production

This Assay consisted of using liquid MRS medium with Durham tubes to detect CO_2 gas, the tubes were inoculated with a 24-hour-old culture of bacteria and then incubated at a temperature of 37C° for a period of 24 to 48 hours. If a gas bubble forms in the Durham tube, this indicates the ability of the bacteria to produce gas from glucose fermentation (Harrigan *et al*.,1998).

3-2-6-16- Sugars Fermentation Assay

The sugar fermentation medium prepared according to (3-2-2-2-9) containing the sugars required in the Assay and prepared according to (3-2-3-12) was inoculated with a portion of the bacterial culture at 48 hours of age and incubated using an Anaerobic Jar at 37C° for 3 to 7 days with colour changes monitored daily and the Assay result was read by changing the colour of the medium from red to yellow, this indicates a positive Assay and this Assay was used to determine the ability of the bacteria to ferment sugars (MacFaddin *et al*., 2000).

3-2-7- VITEK- 2 Compact System – Identification

To obtain a more accurate diagnosis of all laboratory-diagnosed bacterial isolates, diagnosis of the isolates was carried out using the VITEK device technology, and the diagnostic results were interpreted to mean that all isolates that were subjected to the VITEK-2 device Assay were *B. bifidum*, as a special diagnostic card for Gram-positive anaerobic bacteria was used. The card is based on biochemical methods and there are 36 biochemical Assays to measure the use of carbon sources and enzymatic activities. The final results of the diagnosis are available within 6 hours and you can obtain a list in which all the details of the sample are recorded. The card contains 64 holes containing the culture media for each biochemical Assay.

The diagnostic steps were as follows:

1) The ID card was opened from its cover and the model number was recorded on its device register.

2) The bacterial suspension was prepared by taking a sufficient fraction of pure bacterial colonies and adding 3.0 ml of normal saline solution in clear plastic Assay tubes numbered (1).

3) The turbidity of the suspension was compared to the turbidity of the Vitek 2 compact (Densichek TM), which was between 0.5-0.63.

4) The transfer tube connected to the ID card was inserted into Assay tube 1 and rested on the cassette by hand.

5) Transfer the holder to the Vaccum chamber, where the inoculation process occurs inside the device and then transferred to the incubator for incubation at a temperature of 35.5C° and read the result within 6-8 hours.

6) During the incubation period, the device's work was to analyse and store biochemical patterns in an autonomous manner. After the incubation period, the device's software analysed these patterns and then printed a diagnostic report for the card inside the Reader incubator.

3-2-8- Storage *B. bifidum*

Bacterial isolates were preserved in two ways as follows:

3-2-8-1- Short Storage Period

This method consisted of growing bacteria on MRS agar medium kept in tubes with slant surfaces and prepared according to point (b) of paragraph (3-2-2-2-1) by the stabbing method and incubated at a temperature of 37C° for 48 hours so that the growth of bacteria was evident, then They were stored at 4C° and refreshed every twenty days (Holt & Krieg,1984).

3-2-8-2- Long Storage Period

This method consisted of growing the bacteria in the ready-made MRS broth medium mentioned in Table (3-5) prepared according to the manufacturer's instructions and placed in small tubes with tight lids and incubated at 37C° for 48 h. Then Glycerol was added at a concentration of 20%, mixed by Vortex and kept at 20C° (Boonaert & Rouxhet,2000), the information about each sample and the date of preservation should be recorded and renewed every six months to ensure the safety of the samples from damage and death (WHO, 2003).

3-2-9-Molecular Diagnosis of *B. bifidum* Using (PCR)

The PCR technique was used to detect *B. bifidum* from the extract of bacterial cells based on the 16S rRNA gene with a packet size (379) using specialised primers designed specifically for this study to detect the 16S rRNA gene (Catone *et al*.,2024). Five isolates of *B. bifidum* were diagnosed and the primers used in the polymerase chain reaction were as shown in Table (3-6).

Table (3-6) Primers Used in the PCR Reaction

Gene name	Primers Sequence 5′→3′		Primer length	Amplific-ation size (bp)	Annealing Temperatu-re
16S rRNA	F	GAAGAACCTTACCTGGGCTTG	bp21	379	58
	R	ATTACTAGCGACTCCGCCTTC	bp21		

The steps for molecular diagnosis of *Bifidobacterium bifidum* were as follows:

3-2-9-1- Extraction of Deoxyribonucleic Acid (DNA) from *B. bifidum*

Extraction was performed using the DNA Extraction protocol from Promega. The extraction method included the following steps:

1) Sample Preparation where *B. bifidum* isolates were grown in MRS-broth medium and then anaerobically incubated using Anaerobic Jar and placed in Shaker Incubator at 37C° for 24 to 48 hours and then 1.5 ml of growing bacterial culture was withdrawn and dispensed into Eppendorf tubes.

2) The bacterial culture was centrifuged at 13000R/M for 10 minutes to sediment the cells, then the filtrate was removed and the sediment of bacterial cells remained.

3) The filtrate was suspended with 200 µl of Lysozyme and 20 µl of Proteinase K, mixed well with a vortex mixer and incubated for 30 minutes at 37C°.

4) Add 200 ml of GB buffer and mix well with a vortex mixer for 10 seconds, then incubate at 70C° for 10 minutes for Cell Lysis.

5) For DNA Binding, 200 ml of absolute ethanol was added to the cells, mixed well, and a GD Column with a filter was placed in the collection tube. The bacterial suspension with the previous additives was poured into the filter tube and the cell suspension was centrifuged at 13000R/M for 4 minutes, and the collected supernatant was removed and the bacterial cell DNA and other sediment remained in the filter.

6) 400 ml of W1 buffer was added to the GD Column, centrifuged for 1 minute and the filtrate was discarded.

7) 600 ml of the second wash buffer with absolute ethanol was added and the mixture was centrifuged at 13000R/M for 5 minutes.

8) Then a filter tube was placed inside a new Eppendorf tube and the wash tube was discarded. The Elution solution that was preheated in a 70C° water bath for several minutes was added to the filter tube and left for several minutes to allow the DNA of the bacterial cells to disassociate from the filter, then centrifugation was performed for several minutes and then the filter tube was discarded and the Eppendorf tube containing the DNA was retained and then stored at -20C° until use.

3-2-9-2- Polymerase Reaction Techniqe (PCR)

The polymerase chain reaction included the following components shown in Table (3-7):

Table (3-7) Polymerase Reaction Techniqe (PCR) Components

Components		Volume (µl)
Master Mix		12.5
Primers	Forward	10 µM/µl(1)
	Reverse	10 µM/µl(1)
DNA sample		6
Nuclease-Free water		4.5
The total volume of the reaction		25

It also included two types of solutions used in the polymerization reaction:

1- Stock Primers

The Stock Primers solution was prepared according to the instructions of the Korean manufacturer Micro-Gen to obtain a solution with a concentration of 100% µL/picomole and was stored at -20C° until use.

2- Master Mix

I use Master Mix prepared by Intron Biotechnology.

3-2-9-3- PCR Techniqe

Primer solutions were prepared, each primer separately, at a concentration of 10% µL/pmol by adding 10 µL of the stock solution of each primer separately to 90 µL of deionized water, then mixed well and stored in the refrigerator until use. The primer solutions were kept at a temperature of -20C°.

3-2-9-4- Gel Electrophoresis

The electrical relay process requires the following:

1- Solutions Used

- SYBR Green I (SG) stain prepared from Molecular Probes Inc
- Prepare 0.5 X TBE buffer, and 500 ml of distilled water was added to one vial of TBE buffer (X1) according to the manufacturer's instructions.

2- Prepare Agarose Gel 1.5%

The agarose gel was prepared by dissolving 0.75gm of agarose in 500 mL of TBE buffer 0.5X , the agarose was heated to boiling and then allowed to reach a temperature of 45-50C°, then 10 ml of SYBR Green I (SG) stain was added and mixed well, then the tray and comb stabilisation were prepared to make Wells to hold the samples, and the agarose was poured in a light and continuous flow to avoid the formation of air bubbles and left to solidify at laboratory temperature, then the comb was gently removed, and the gel was transferred from the mould to the migration basin for the electrophoresis process.

3-2-9-5- Products of Replication (DNA)

The replication products were detected by transferring the samples onto an agarose gel after completing the migration process by exposing them to an ultraviolet source and estimating the molecular sizes of the replication pieces and comparing them with the location of the bands for the size guide and the stages with the replication products (Biswas,2024).

3-2-9-6- Polymerase Chain Reaction (PCR) Program

Optimization of amplification for the selected gene was performed under the following conditions as in Table (3-8).

Table (3-8) Polymerase Chain Reaction (PCR) Program

Steps	Temperature (C°)	Time	Cycles
Initial Denaturation	95	5 min	1
Denaturation	95	30 sec	30
Annealing	57±5	30 sec	
Extension	72	30 sec	
Final extension	72	7 min	1

3-2-10-Assaying the Ability of *Bifidobacterium bifidum* to Produce Biosurfactant

The ability of the bacteria to produce BS was investigated by the following two Assays:

3-2-10-1- Diffusion of Oils Assay

This Assay was carried out by pouring 30 ml of distilled water into a sterile petri dish and then adding 1 ml of Coconut Oil and Gingely Oil in the centre of the dish containing distilled water and then adding 20 µl of bacterial culture in the centre. The result of this Assay is read by observing the displacement of the oils and their dispersion in the water (Anandaraj & Thivakaran,2010).

3-2-10-2- Emulsification Index E24 Assay

The emulsification index Assay was performed according to (Cooper& Goldenberg,1987) by mixing equal amounts of liquid bacterial culture and kerosene and mixing well with a vortex mixer for 2 minutes and then left for 24 hours to evaluate E24.If a foam-like emulsified layer appears above the solution, this indicates that the bacteria have the ability to emulsify fats, and the higher the foam, the stronger the emulsification.

3-2-11- Extraction of Biosurfactant Produced from *Bifidobacterium bifidum*

Biosurfactant extraction was performed according to the method of (Lievin *et al*., 2000), by reculturing *B. bifidum* for activation in MRS-L-cysteine medium and incubated anaerobically in (Anaerobic Jar) in shaking incubator 120\rpm at 37C° for 48 hours , after the incubation period, the bacterial culture was transferred to tubes and placed in a Cooling Centrifuge at 4C° at 1000 R/M for 30 minutes, the bacterial solution was then filtered through a Millipore filter with a diameter of 0.22 micrometers and the precipitate containing the bacterial cells was obtained from the filtrate and kept at 4C° until use, and the cells were washed twice with deionised water , then the cells were re-suspended in a solution of PBS with a pH of 7.0 and left for 3 to 4 hours at laboratory temperature with gentle shaking with a Magnetic Stirrer, which allows the release of the Biosurfactant , the solution was put back in the centrifuge to get rid of cell residues, and the solution was filtered with a Millipore filter paper with a diameter of 0.22 micrometers, to separate the filtrate from the sediment containing the Biosurfactant , then used a Lyophilizer to dry the Biosurfactant to obtain a white to pale yellow powder. It was stored at a temperature of 4C° until use.

3-2-12- Cell Culture of the Colorectal Cancer Cell Line [HRT-18]

The colorectal cancer cell line [HRT-18] was obtainee from the Center for Natural Product Research and Drug Discovery, Department of Pharmacology, Faculty of Medicine University of Malaya, Kuala Lumpur, preserved in liquid nitrogen, concentration of 1×10^4 to 1×10^6 cells/ml, to sustain and grow the cells and conduct laboratory Assays on them, taking into account methods. Sterilization required in cellular agriculture is moist heat sterilization and membrane filters.

3-2-13- Cell Culture of the Fibroblast [HDFn]

Fibroblast cell line represents the most common cell type in connective tissue and is the structural framework of animal tissues. These cells produce a variety of products including Elastin, Collagen Types I, III and IV, Proteoglycans, Fibronectin and Laminins, glycosaminoglycans, metalloproteinase, and prostaglandin, also called fibroblasts or fibroblasts Juvenile cells were obtained from the same source that provided the cell line [HRT-18], concentration of 1×10^4 to 1×10^5 cells/ml cells/ml

3-2-14- Media and Solutions for Cell Culture

Cell culture media and solutions were prepared according to the source (Freshney *et al*.,2015).

3-2-14-1- Fetal Bovine Serum (FBS)

Fetal bovine serum (FBS) was used and heat inactivated at a temperature of up to 56C° for 30 minutes and then added to the culture medium.

3-2-14-2- Phosphate – Buffer Saline

This solution was prepared in accordance with (3-2-3-3) and following the manufacturer's instructions.

3-2-14-3-Trypsin Solution

This solution was prepared by dissolving 1 g of trypsin powder in 100 ml of PBS prepared according to (3-2-3-3) and sterilised with fiter paper 0.22 micrometers and placed in 10 ml tubes and kept at -20C° until use.

3-2-14-4- Ethylene Diamin Tetraacetic Acid (EDTA)

This solution was prepared by dissolving 1 g of Ethlene Diamine Tetra Acetic acid (EDTA) in 100 ml of PBS solution prepared according to paragraph (3-2-3-3), sterilised using Autoclave for 10 minutes, placed in 10 ml tubes and stored at 4C° until use.

3-2-14-5- Trypsin-EDTA

This solution was prepared by mixing 20 ml of trypsin solution prepared according to (3-2-14-3), 10 ml of EDTA solution prepared according to (3-2-14-4) and 370 ml of phosphate buffered saline (PBS) solution prepared according to (3-2-3-3), then the mixture was placed in a glass jar and kept at 4C° until use.

3-2-14-6- Sodium Bicarbonate Solution $NaHCO_3$

This solution was prepared by dissolving 4 g of $NaHCO_3$ in 100 ml of distilled water and sterilized using an Autoclave at a temperature of 121C° for 15 minutes, then stored at 4C° until use.

3-2-14-7- Preparation of RPMI-1640 Culture Medium

This medium is a ready-made nutritional medium extracted from bovine serum, it was prepared according to the manufacturer's instructions. Then 10 ml of bovine serum was added to it to multiply the proteins and growth factors, then the solution was transferred to clean, sterile glass bottles and placed in an incubator at a temperature of 37C° to ensure no contamination until use.

3-2-14-8- Preparation of Culture Serum Free Media (SFM)

This medium was prepared in the same way as the RPMI-1640 medium described in paragraph (3-2-14-7), but without adding fetal bovine serum to the culture medium.

3-2-14-9- Preparing the Freezing Medium

This medium was prepared by adding 6 ml of bovine serum-free medium (SFM) prepared according to paragraph (3-2-14-8) and 3 ml of fetal bovine serum (FBS) prepared according to paragraph (3-2-14-1) and 1 ml of DMSO, which is added in successive drops with continuous stirring, then the medium is stored at a temperature of -20C° during use, and it is preferable to prepare it immediately when starting the freezing step cancer cell line.

3-2-14-10- Preparation of MTT Stain

This stain was prepared by dissolving 50 mg of MTT Stain powder in 100 ml of (PBS) solution prepared according to paragraph (3-2-3-3) to obtain a concentration of 5 mg/ml. Then the solution was sterilized with filter paper with a diameter of 0.22 micrometers, then with filter paper with a diameter of 0.45 mm, then placed in clean, sterile, opaque glass bottles away from sunlight , the bottles were stored at a temperature of 4C°, Until use (Meerloo *et al*.,2011).

3-2-15- Investigating the of Anti-tumor Effect of Biosurfactant Agenton human Colorectal Cancer Cell Line HRT-18

To investigate the antagonism of the Biosurfactant, this was done through the following:

3-2-15-1- Maintenance and Preparation of [HRT-18] Cell Line

To maintain and prepare the cancer cell line, rely on the method (Freshney, 2015) as follows:

1) Take out the incubated cancer cells in liquid nitrogen freezer at

-210C° for reactivation and place the tubes containing the cells in a 37C° water bath to thaw them and then transfer them to culture flasks containing RPMI-1640 culture medium prepared according to (3-2-14-7).

2) The cells were left in a humid environment for 24 hours to allow full spreading in the culture medium. Then, they were transferred to an incubator containing CO_2 at a temperature of 37C°.

3) After the incubation period and confirmation of cell growth without contamination, subcultures are prepared for their proliferation.

4) The cells were then examined by an inverted microscope to confirm the viability of the cells, the absence of contamination and their growth to the required number of 600-700 cells/ml, i.e. the percentage of cell proliferation exceeded 80% in the culture medium.

5) Remove the RPMI-1640 medium and wash the adherent cells with PBS two to three times.

6) Add 1 to 2 ml of Trypsin-EDTA solution prepared according to (3-2-14-5) to the cultured cells to obtain single cells and then incubate the flask at 37C° for 2 minutes to separate the cells from the surface of the flask.

7) To completely stop the activity of trypsin, the RPMI-1640 culture medium was added again.

8) The cells were transferred to special tubes and placed in a centrifuge at 200 R/M for 10 minutes to sediment the cells and remove the filtrate, then the cell suspension was distributed in other tubes containing RPMI-1640 medium.

3-2-15-2- Subculture of HRT-18 Cancer Cell Line

Secondary culture is done when required to separate the adherent monolayer cells at the bottom of the primary culture tubes and for the purpose of cellular counting of cells. Secondary culture is based on the method (Phelan & May, 2017), by using the proteolytic enzyme Trypsin. This process is done in the following steps:

1) Discard the culture medium when cell growth reaches a monolayer (exponential phase).

2) The cells were washed with 3 ml of PBS.

3) Add 1 to 2 ml of Trypsin-EDTA solution to submerge the monolayer while continuously stirring the flask 4 to 5 times to completely submerge the cells.

4) The flask was incubated in the incubator at a temperature of 37C° for a period ranging from 2 to 10 minutes to allow the cells to detach from the inner surface of the flask (the length of time of incubation for the detachment process to occur depends on the type of cancer cell line).

5) Note that there should be no excessive use of the Trypsin-EDTA solution, which could cause damage to the cells, so it is necessary to examine them every few minutes until the secondary cultivation process is completed.

6) The cells were examined with an inverted microscope to ensure that all cells had detached and then resuspended with occasional shaking of the flask.

7) Remove Trypsin-EDTA solution from the culture medium by adding Fetal bovine serum (FBS) at 5-10%.

8) The required volume of suspension cells was then transferred to a new flask and supplemented with the required volume of culture medium. The cells were then incubated in the incubator at 37C° to renew, stabilize and restore its activity approximately 24 hours, and this process may be repeated depending on the growth requirements of the cell line.

3-2-15-3- Refrigeration of Cell Line

The HRT-18 cell line was frozen according to the source (Yang *et al*.,2019) as in the following steps:

1) The monolayer cell culture flask (exponential phase) was washed twice with 5 ml of PBS and then 3 ml of Trypsin-EDTA solution was added.

2) The flask was incubated in the incubator at 37C° to allow the cells to disintegrate into single cells after monolayer cell detachment with the help of light stirring on the sides of the flask.

3) Cells were transferred to special sterile 15ml tubes and centrifuged at 800 rpm for 10 minutes.

4) After centrifugation, the supernatant was removed and the cells were resuspended by adding 1 ml of freezing medium prepared according to (3-2-14-9).

5) The flask was left for 10 minutes at laboratory temperature and then transferred to a freezer at -80C° for 24 hours and then stored in a liquid nitrogen freezer for long-term preservation.

3-2-16- Preparation of Different Concentrations of (BS) and the Chemotherapy FOLFOX.

Different concentrations of Biosurfactant (BS) and chemotherapy (FOLFOX) were prepared, The FOLFOX chemotherapy regimen is not available as a ready-mixed, pre-prepared mixture for research or clinical use. Instead, its three components are obtained separately and mixed according to specific protocols. 0.01g of the dry compounds were dissolved in serum-free medium (SFM) prepared according to section (3-2-14-8). Then five serial concentrations were made, diluted in half to reach the lowest concentration, which are as follows (400, 200, 100, 50, 25) µg/ml.

3-2-17-Methylthiazolyldiphenyl-tetrazolium (MTT) Assay to Assay for Cytotoxicity

The MTT (3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) assay is a non-radioactive colourimetric assay used to measure cell viability in response to a variety of cytotoxic stimuli (Stockert *et al*.,2018). The colour change

of the MTT stain from yellow to purple is caused by the enzyme Mitochondrial (NADH dehydrogenase) if the cells are alive

The MTT assay is based on the colour transformation and formation of dark-coloured insoluble formazan through the reduction of water-soluble tetrazolium, which helps in knowing the metabolic activity of living cells and detecting cell growth and proliferation by measuring the optical density (absorbance), thus using the MTT assay provides an assessment of cell development after treatment with the Biosurfactant (Buch *et al*.,2012).

3-2-17-1- Principle

The purpose of this assay was to measure the cell viability and assess the cytotoxicity of *B. bifidum*-derived Biosurfactant, 96 wells were used with different concentrations and replicates for each well.

Since the principle of the Assay is related to mitochondria, an increase or decrease in the number of cells is closely related to the activity of the mitochondria, this activity is reflected by converting the pale yellow-coloured tetrazolium salt (MTT stain) into Formazan stains that form purple crystals that can be dissolved for homogeneous measurement after an incubation period of 2 to 4 hours, which helps in knowing the increase and decrease in the number of living cells. By measuring the concentration of Formazan, which is reflected by the optical density (absorbance) using a microplate reader at a wavelength of 570 nm and the Assay result is read by the colour where the darker the colour, the higher the number of live cells (Meerloo *et al*.,2011).

In the present study cytotoxicity measurement was performed in three replicates and the IC50 value was calculated, and the cytotoxic effect of different concentrations of BS extracted from B. bifidum on colorectal cancer cell line HRT-18 was determined and compared with untreated cells and cells treated with chemotherapy (Genetics Home Reference - G H R,2018).

3-2-17-2- Contents of the (MTT) Kits Assay

The MTT assay kit consists of

1) MTT solution (1ml x 10 glass vials)

2) Solubilisation solution (50 x 2 bottles)

3-2-17-3- Procedure

The method is based on the source (Amritha *et al.*,2015) for vitality detection and is as follows:

1) Cells were grown at approximately 1×10^4 cells per well according to (3-2-15-1). The cell suspension was placed in a 96-well plate with a flat bottom, it was covered with sterile Para film, shaken gently,200 µl of culture medium was added to each well, and incubated at 37C°for 24 hours to form a confluent monolayer.

2) After the end of the incubation period, the culture medium was removed and the diluted concentrations of BS prepared according to (3-2-16) (400, 200, 100, 50, 25) µg/ml were added to the plate pits to investigate their efficacy as a treatment.

3) Three replicates were used for each concentration including negative control (cells + medium) and positive control (cells + FOLFOX treatment).

4) After 48 hours of treatment, cell viability was measured by adding 28 microlitres at a concentration of 2 mg/ml of MTT solution.

5) After incubating the cells at 37C° for 1.5 hours, the MTT stain was removed from the plate after intracellular Formazan crystals appeared.

6) A DMSO solution with a volume of 130 µl was added in each well to dissolve the resulting Formazan crystals.

7) The plates were then incubated at 37C° for 15 minutes with constant shaking to dissolve the purple crystals.

8) Absorbance was measured by a microplate reader at a wavelength of 575 nm and the assay was performed for all three replicates.

9) The rate of cell inhibition (cytotoxicity) was calculated according to the following equation :

Cell viability = $\frac{absobance\ of\ treated\ cells}{absobance\ of\ non\ treated\ cells} \times 100$

Cytotoxicity = 100 – cell viability

10) The result of the reader microplate (OD) was subjected to statistical analysis to determine the concentration of BS needed to kill or inhibit the growth of half of the cancer cells according to the following formula:

Viability = $\frac{Optical\ density\ of\ the\ sample}{Optical\ density\ of\ the\ control} \times 100$

Whereas :

Viability %: Percentage of cell viability

3-2-18- High Content Screening (HCS) Assay to Assess Cytotoxicity

It is known as High Content Analysis (HCA) or cellomics, it is an advanced technique used in drug discovery and biological research and to analyse the effects of chemical compounds, genetic materials or other biologically active molecules on cells which alters the cell's phenotype in a desirable way (Lin *et al*., 2020). HCS also provides a more detailed and multifaceted analysis of cellular responses (Giuliano & Haskins,2010).

This Assay involved measuring changes in the physiological functions of the cells (Vivek,2008) and comparing them with untreated and chemotherapy-treated cells. This Assay was conducted at the University of Malaya/Department of Pharmacy/Centre for Investigation of New Drugs in Malaysia.

The fold of change values were calculated based on the following equation:

$Fold\ of\ Change = \frac{Parameters}{Control}$

3-2-18-1- Contents of the (HCS) kits

As shown in Table (3-9).

Table (3-9) Components of the (HCS) Kit

Materials	Concentrations
Cytochrome C Primary Antibody	75 µl
Dylight ™ 649 Conjugated Goat- Mouse Antibody	75 µl
Mitochondrial Membrane Potential Stain	1 µl
Permeability Stain	25 µl
Hoechst Stain	30 µl
Wash Buffer (10 X Duldeccos PBS)	100 µl
Permeabilization Buffer (10 X Duldeccos PBS with 1% Triton X100)	100 µl
Blocking Buffer (10X)	85 µl
Thin Plate Seal Assembly	7/ pack

3-2-18-2- Solution preparation

The solutions required for the HCS Assay were prepared as follows:

1) 1X Wash Buffer

This solution was prepared by taking 20 ml of 10X Wash Buffer, adding 180 ml of distilled water and keeping the solution at 4C° for 7 days.

2) Fixation Solution

It is Prepare just before use by taking 3 ml of 16% Paraformaldehyde solution and adding to 9 ml 1X Wash Buffer.

3) 1X Permeabilization Buffer

It is Prepared by taking 15 ml of Permeabilisation Buffer and adding it to 13.5 ml of Wash Buffer and kept at 4C° for 7 days.

4) 1X Blocking Buffer

It is Prepared by taking 5 ml of 10X Blocking Buffer and adding it to 44 ml of Wash Buffer and kept at 4C° for 7 days.

5) Primary Antibody Solution

It is Prepare the solution immediately before the examination by taking 15 µl of Cytochrom C Primary Antibody and adding it to 6 ml of 10X Blocking Buffer.

6) Secondary Antibody Staining Solution

It is The buffer was prepared prior to the assay by taking 0.6 µl of Hoechst Stain and 12 µl of Dylight ™ 649 Conjugated Goat- Mouse Antibody and diluted to 6 ml of 1X Blocking Buffer.

7) Live Cell Staining Solution

It is Prepared by taking 117 µl of DMSO by adding it to Mitochondrial Membrane Potential Stain to prepare 1 ml of M Stock solution, then taking 2.1 µl of Permeability Stain and 21 µl of Mitochondrial Membrane Potential Stain and adding it to 6 ml of complete medium.

3-2-18-3- Procedure

1) Concentrations of BS extracted from *B. bifidum* , from The lowest concentrations of BS which showed efficacy against HRT-18 cell line based on MTT assay results, were prepared, 50 µl of different concentrations of BS were added to the wells containing the HRT-18 colorectal cancer cell line prepared according to (3-2-151-) at 3 replicates per concentration, and 50 µl of culture medium was added to the negative control and positive control wells, then the cells were incubated at 37C° for 24 hrs.

2) Then, 50 µl of live cell staining solution was added to each well, and the cells were incubated at 37C° for 30 minutes.

3) The staining solution and medium were quietly withdrawn, then 100µl/well of fixation solution was added and the plate was incubated at laboratory temperature for 20 minutes.

4) The fixation solution was quietly withdrawn, then 100 µl/well of wash buffer was added for 10 minutes.

5) The wash buffer was withdrawn, then 100 µl/well of permeabilization buffer was added and the plate was incubated at laboratory temperature for 10 minutes.

6) The wash buffer was withdrawn, then 100 µl/well of blocking buffer was added and the plate was incubated at laboratory temperature for 15 minutes.

7) The blocking Buffer was withdrawn, then 50 µl/well of primary antibody solution was added and the plate was incubated away from light at laboratory temperature for 60 minutes.

8) The primary antibody solution was withdrawn and the plate was washed three times with 100 µl/well of wash buffer solution.

9) The wash buffer was removed, 50µl of staining solution (secondary antibodies) was added to each well, and the plate was incubated away from light at laboratory temperature for 60 minutes.

10) The secondary antibody staining solution was withdrawn and the plate was washed three times with 100 µl per well in the wash buffer.

11) Then 100 µl was added to each well of the wash buffer, the plate was closed tightly and then read by the Array Scan HCS Reader.

3-2-19- Assay Caspase 9 and Caspase 8

The Caspase assay is an optical technology (Illuminacea), from Promega that measures Caspase activity, which plays an important role in maintaining cell homeostasis in terms of its growth, reproduction, differentiation, and programmed death , Caspase activity ensures the degradation of cell components in a controlled manner, leading to cell death with minimal effects on surrounding tissues, Caspase 9 and 8 proteins are linked to programmed cell death, as they follow two paths: the internal path, which is responsible for Caspase 9, and the external path, which is responsible for Caspase 8 (Rongvaux *et al*.,2014 ; Thornberry *et al*.,2000 ; Costantini *et al*.,2002).

Perform this Assay to determine which caspase pathway is responsible for the programmed death of the RTH-18 colorectal cancer cell line when treated with

different concentrations of Biosurfactant (BS) extracted from *B. bifidum.* It was conducted at the University of Malaya / Department of Pharmacy / Centre for Investigation of New Drugs in Malaysia.

3-2-19-1- Components of the 8 Caspase-9 Assay Kit

1) Caspase-Glo® 9 and 8 Buffer 10×1 mL.

2) Caspase-Glo® 9 and 8 Substrate (lyophilised).

3) MG-132 Inhibitor 30µL.

All components were stored at -20C°.

3-2-19-2- Procedure

1) HRT-18 cancer cell line cells were prepared according to paragraph (3-2-15-1), and 100 microliters of them were added to a 96-well plate and incubated at 37C° for 24 hours.

2) After the incubation period, the culture medium was removed and 50 µl of prepared concentrations of (BS) extracted from *B. bifidum* bacteria were added to the pits, which are (200, 100, 50, 25)µg/ml, at (3) replicates for each concentration, with (3) replicates for the negative control pit and the positive control pit, then the cells were incubated at a temperature of 37C°, For 30 minutes.

3) After the incubation period, the plate is allowed to equilibrate at laboratory temperature for a short period, then 100 µl of the components of the Caspase-Glo® Reagent kit are added to each well.

4) The contents of the plate were mixed vortexally using an Orbital Shaker at (300-500) R/M for a period ranging from 0.5 to 2 minutes.

5) The plate was then incubated for 30 minutes at laboratory temperature, after which the absorbance was measured using an ELISA device at 405 nm.

3-2-20- Diphenyl Picrylhydrazyl Radical Scavenging (DPPH) Assay

The diphenyl-1-picrylhydrazyl-2,2 (DPPH) assay is one of the most common colorimetric Assays for determining free radical scavenging in compounds and extracts. This method is accurate, easy to perform, and economical, and provides an examination of the general activity of antioxidants. When DPPH reacts with an antioxidant compound, its free radical property is lost because the free radicals will bind to biological compounds (That is, it removes electrons from other compounds) and its color changes from violet to yellow (Zeynep *et al*., 2017).

3-2-20-1-Assay Kit Components

1) DPPH Reagent × 1

2) Trolox Standard × 1

3) Assay Buffer 11ml × 1

3-2-20-2- Procedure

1) HRT-18 cancer cell line cells were prepared according to paragraph (3-2-15-1), and 100 microliters of them were added to a 96-well plate and incubated at 37C° for 24 hours.

2) Weigh 10 mg of DPPH and add it to a 100 ml volumetric measuring vial and add 100 ml of solvent to the vial , (methanol or ethanol) are the commonly used solvents for DPPH solution.

3) After the incubation period, the culture medium was removed and 507 µl of prepared concentrations of BS (200,100,50,25)µg/ml (3 replicates for each concentration), prepared concentrations of Ascorbic acid (200,100,50,25)µg/ml (3 replicates for each concentration) and control (DPPH + solvent) were added to the pits and the cells were incubated at 37C° for 1 hr.

4) After the incubation period, the plate was allowed to equilibrate at laboratory temperature for a short time, then 750 µl of DPPH solution was added to each hole

and DPPH was prepared immediately prior to use and the plate was allowed to equilibrate at laboratory temperature.

5) The DPPH solution was removed and 507µl of Trolox Standard was added and the plate was left to stabilise at laboratory temperature for a short time.

6) Assay Buffer was then added and the contents of the plate were vortexed using an Orbital Shaker at 300-500R\M for 0.5-2 minutes and incubated for 60 minutes at laboratory temperature.

7) Measurement of optical density (OD) at a wavelength of 450 nm by ELISA.

3-2-21- Reactive Oxygen Species (ROS) Assay

The production of reactive oxygen species (ROS) is one of the main causes of cell damage during energy release. ROS can damage lipids, DNA, and RNA, as well as oxidize polyunsaturated fatty acids in lipids (lipid peroxidation), amino acids in proteins, and specific enzymes through oxidative inactivation by co-oxidants. This process contributes to programmed cell death (apoptosis), (ROS) play an important role in all body functions (Nosaka & Nosaka,2017).

(ROS) are unstable molecules that contain oxygen and are byproducts of the normal metabolic process of oxygen , they play a role in cell signaling and balance, are used to investigate immune responses, monitor the balance between reactive oxygen species (ROS) and antioxidants, or study their role in cancer development (Samuni *et al*.,2021).

These molecules are highly reactive and come in many different forms, examples include H2O2 (hydrogen peroxide), NO (nitric oxide), O_2- (oxide anion), peroxynitrite (-ONOO) and (hydrochlorous acid) (HOCl) hydroxyl radical (OH^-)[1] (Holmström & Finkel,2014).

The purpose of this assay is to provide a reactive oxygen species (ROS) screening protocol to study the photoactivity of chemicals (Onoue *et al*., 2008), which helps in identifying oxidative stress that may be responsible for tumour development or response to treatment, monitoring the effectiveness of treatments

and identifying drug resistance, ROS screening was conducted at University of Malaya / Department of Pharmacy / New Drug Investigation Centre in Malaysia.

3-2-21-1- Assay Kit Components

1) ROS Assay Buffer

2) ROS Label (Staining\Dihydroethidium (DHE), Hoechst)

3) ROS Inducer , ROS inhibitor

3-2-21-2- Procedure

1) The reagents were prepared according to the manufacturer's instructions and stored at the temperatures mentioned in the instructions, preferably immediately before use.

2) HRT-18 cancer cell line cells were prepared according to paragraph (1-15-2-3) at a density not exceeding 5×10^5 cells/ml. They were placed in a 96-well plate and incubated at 37C° for 24 hours.

3) After the incubation period, the culture medium was removed and 507 µl of the prepared concentrations of (BS) were added to the pits, which are (200, 100, 50, 25)µg/ml, at (3) replicates for each concentration, with (3) replicates for the negative control pit to which 50 µl of the culture medium was added, and (3) replicates for the control pit. positive, then the cells were incubated at 37C° for 24 hours.

4) After the incubation period, the plate was allowed to equilibrate at laboratory temperature for a short period, then the pit content was removed, and 100 microliters of ROS Inducer solution was added to each pit and incubated at 37C° for 24 hours.

5) ROS Inducer was removed from the pits and 100 µl of ROS Inducer was added to each pit and incubated at 37C° for 30 min.

6) The reaction stain was added to all the wells containing the cells, and the contents of the plate were mixed vortexally using an Orbital Shaker at 300-50 R\M for 5 minutes and incubated at a temperature of 37C° for 30 minutes away from light.

7) Absorbance values were measured using a luminometer, which is an instrument that measures photons of visible emitted light coming from a sample, via a photomultiplier tube.

3-2-22- Investigation of *NOX4* Gene Expression in Colorectal Cancer Cell Line [HRT-18]

To investigate the gene expression of *NOX4* gene in colorectal cancer cell line [HRT-18] by the following steps:

3-2-22-1- Quantitative Reverse Transcription Real-Time PCR (RT-qPCR) Assay

Quantitative Reverse Transcription Real-Time PCR (RT- qPCR) assay was performed to measure the quantitative levels of mRNA to indicate the amount of gene expression of the *NOX4* gene in the colorectal cancer cell line [HRT-18] cells. The gene (*GADPH*) was used as a standard regulator to calculate gene expression. This assay was performed according to the method of (Xiao-Lu *et al*., 2017) as follows:

3-2-22-1-1- Total RNA Extraction by TRIzol™

Total RNA was extracted using the Trizol kit manufactured by the Korean Pioneer Company and was performed according to the company's instructions as follows:

1) After treating the [HRT-18] colorectal cancer cell line with different concentrations of the Biosurfactant extracted from *B.bifidum* prepared according to paragraph (3-2-16) , 250 microliters of it was taken and distributed in 1.5 ml tubes , and 1 ml of Trizol solution was added to it and mixed well for 2 minutes by Vortex.

2) 200µl of Chloroform alcohol was added to each tube and shaken for 15 minutes by Vortex and the tubes were kept in the refrigerator for 10 minutes.

3) The tubes were placed in a centrifuging device for 1 minute at 13000 R\M.

4) The supernatant was drawn off using a micropipette and placed in new Eppendorf tubes. An equal amount of Isoprobanol alcohol was added to it. The tubes were shaken gently by hand 4 to 5 times and the tubes were kept at a temperature of -20C° for 10 minutes.

5) The tubes were placed in a centrifuge for 10 minutes at 13000 R\M and the supernatant was discarded leaving the precipitate of cells.

6) 1 ml of Ethanol alcohol 80% , was added to the precipitate and mixed using a Vortex device. The tubes were then centrifuged for 5 minutes at 13000 R\M , The supernatant was discarded and the precipitate was allowed to dry at laboratory temperature for 10 minutes.

7) The precipitate was preserved by adding 50 ml of Diethyl pyrocarbonate (DEPC) water and placed in a water bath at 55-60C° for 10 minutes, after which the extracted RNA was preserved at -70C°.

3-2-22-1-2- Measure the Concentration and Purity of Total RNA

The concentration and purity of the extracted RNA ng/ul was investigated using a Nanodrop spectrophotometer and the purity of the RNA was measured by reading the absorbance at 260-280 nm as follows:

1) After turning on the Nanodrop spectrophotometer, the device was zeroed by placing 2 microliters of Free nuclease water using a sterile micropipette on the surface of the substrate, then the substrate was cleaned with blotting papers to measure the samples.

2) The OK key was pressed to start the RNA concentration measurement process, 1µl of each extracted RNA sample was added, and the meter substrate was cleaned each time to measure the other samples.

3) The purity of the extracted Total RNA samples was determined by reading the absorbance of the Nanodrop spectrophotometer at a wavelength of (260-280 nm). The RNA is considered pure when the absorbance is (1.2-2).

3-2-22-1-3- DNase 1 Treatment

To remove DNA residues during the extraction process, DNase enzyme was added to all extracted Total RNA samples according to the method of the enzyme kit shown in the table (3-10). The mixture was then incubated in an incubator at 37C° for 30 minutes, then 1 microliter of EDTA was added to the samples and then kept in a water bath at 65C° for 10 minutes to activate the enzyme activity.

Table (3-10) DNase Enzyme Kit

Mix	Volume
Total RNA 100 ng/ul	10 ul
DNase 1 enzyme	1 ul
10X buffer	4 ul
DEPC Water	5 ul
Total	20 ul

3-2-22-1-4- cDNA Synthesis

The method of manufacturing cDNA complementary to cDNA from extracted RNA samples was used by Accupower Rockscript RT Premix Kit provided by Pioneer Korea and used for the purpose of converting RNA to cDNA. This process was carried out according to the method of operation of the kit as shown in the following table (3-11).

Then the components of the RT master mix in Table (3-11) were added to the cDNA kit tubes containing the Reverse transcription enzyme and then all the tubes were transferred to a Vortex centrifuge at 3000 R\M for 3 minutes.

Then the tubes were transferred to the Thermo Cycler and the thermal conditions for the cDNA synthesis process were applied according to the method of the kit

shown in Table (3-12). After that, the samples were stored at a temperature of -20C° until they were used in the RT-PCR Assay.

Table (3-11) Reverse Transcription Reaction

RT master mix	Volume
Total RNA 100 ng/ul	10 ul
Random Hexamer Primer 10 pmo	1 ul
DEPC water	9 ul
Total	20 ul

Table (3-12) Thermal Conditions for cDNA Synthesis

Step	Time	Temperature
cDNA synthesis (RT step)	1 Hour	50 C°
Heat inactivation	5 Minutes	95C°

3-2-22-1-5- Quantitative Real-Time (PCR)

Accupower 2×Green star qPCR Kit provided by Pioneer Korea was used to perform qPCR of the study cDNA samples containing SYBR Green stain which reacts with the amplified genes in the RT-PCR device as follows in Table (3-13).

Table (3-13) Preparation of qPCR Reaction Mixture for Target Gene (*NOX4*) and Standard Regulator Gene (*GAPDH*)

qPCR Pre Mix	Volume
cDNA Template	2.5 µL
Primers 10 pmol / gene-F	1.25 µL
Primers 10 pmol / gene-R	1.25 µL
2× Green star master mix	25 µL
DEPC Water	20 µL
Total	50 µL

The components mentioned in Table (3-13) were added to the special tubes for qPCR, then all tubes were placed in the Vortex centrifuge at a speed of 3000 rpm for 3 minutes, and the plate was transferred to the (Miniopticon Real-Time PCR device BioRad.USA),the qPCR Thermocycler conditions were applied to all genes according to the method of operation of the kit as in Table (3-14).

Table (3-14) Optimal Temperature Conditions for qRT-PCR Stages for *NOX4* and *GAPDH* Genes

qPCR Pre Mix	Volume
cDNA Template	2.5 µL
Primers 10 pmol / gene-F	1.25 µL
Primers 10 pmol / gene-R	1.25 µL
2× Green star master mix	25 µL
DEPC Water	20 µL
Total	50 µL

3-2-22-1-6- Real-Time PCR Data Analysis

To analyze the data, the method developed by (Livak & Schmittgen, 2001) called (The Δ CT Method Using a reference gene) $2^{-\Delta\Delta Ct}$ was used for the purpose of analyzing data resulting from quantitative real-time polymerase chain reaction , this method depends on extracting the Relative Quantitative after correcting it so that it becomes biologically meaningful to calculate the (fold change) in the target genes of the current study of *NOX4* gene using the conservative gene *GAPDH* as a standard regulatory gene for correction.

The threshold cycle number (Ct) values of the target gene were corrected with the Ct values of the conservative gene by quantitative expression ratio levels (fold change) levels based on the Livak method so that one of the study samples is a Calibrator representing Control samples , then all corrected Ct Values are divided

with Calibrator to extract the relative expression levels (fold change) , then use the ΔCT method with the correction gene as in the following steps :

1) The Ct value of the Reference gene (ref) was corrected for the gene targeting the standard sample and the gene targeting the study sample as shown in the following two equations :

$$\Delta Ct\ [Assay] = Ct\ [target\ ,\ Assay] - Ct\ [Ref\ ,\ Assay]$$

$$\Delta Ct\ [Control] = Ct\ [target\ ,\ Control] - Ct\ [Ref\ ,\ Control]$$

2) The ΔCt value of the target gene of the study sample was corrected from the ΔCt value of the target gene of the control sample as shown in the following equation:

$$\Delta\Delta Ct = \Delta Ct\ [Assay] - \Delta Ct\ [Control]$$

3) The fold change ratio of gene expression levels is calculated as shown in the following equation:

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Whereas **:**

Ct [target , Assay] Indicates cycle threshold (target gene, treated sample)

Ct [Ref , Assay] Indicates cycle threshold (conservative gene, treated sample)

Ct [target , Control] Indicates cycle threshold (target gene, control sample)

Ct [Ref , Control] Indicates cycle threshold (conservative gene, control sample)

3-2-23- Statistical Analysis

The data were statistically analyzed according to the A one way analysis of variance (ANOVA) Assay (Tukey's Assay) and to determine the statistical and significant differences, the statistical analysis program (SPSS) was used. The differences were considered statistically significant and highly significant at the level of ($*p \leq 0.05$ and $**p \leq 0.01$), and the data were expressed as (Mean ± SD) using (Graph Pad Prism) version 9 (Graph Pad Software Inc., Jolla, CA).

Chapter Four

Results and discussion

4-Results and Discussion

4-1- Isolation and Identification

A total of of 50 samples were collected from different sources, including imported and local dairy products, milk cow's, and human breast milk , the study samples included 30 (60%) imported and local dairy products gave 2 (40%) samples were positive for Bifidobacterium bifidum , collected of the cow's milk 15 (30%) gave 2 (40%) gave positive results , from breast milk 5 (10%) this gaved 1 (20%) positive sample as shown in Table (1-4).

Table (4-1) Numbers and Percentages of *B. bifidum* Samples from Different Isolation Sources

N	Insulation sources	Number of samples	Percentage	Number of positive samples	Percentage
1.	Imported and local dairy products	30	%60	2	%40
2.	Cow's milk	15	%30	2	%40
3.	Breast milk	5	%10	1	%20
Total		50	%100	5	%100

Bifidobacterium bifidum was isolated from dairy products, cow's milk and human milk after purification of the isolates on MRS-L-Cystiene-HCL medium , the isolates were identified by the morphological characteristics of the growing colonies, which appeared as cream-coloured, convex, circular colonies resembling dewdrops , It has a smell similar to the smell of fermenting milk as in the picture (4-1).

Microscopic characteristics were identified by staining with Gram stain, and the result was positive for Gram stain , it grows under anaerobic conditions in the form of short rods, singly or in binary aggregates, and does not form spores, as shown in Table (4-2).

It was identified by biochemical Assays and was negative for KOH Assay negative for Oxidase and Catalase, non-producer of Indole from tryptophan, negative for Citrate consumption, negative for Urease, non-gelatin and Starch degrader, negative for Ammonia production from arginine , negative for Nitrate reduction, non-degradable for casein, Lecithin and Lipase, non-Motile, Grows at temperatures ranging between (35-45C°) and does not Grow at temperatures ranging between (5-15C°) and negative for Gas production as shown in Table (4-3).

It was also diagnosed based on the fermentation of sugars. It ferments Glucose, Fructose, Galactose, Sucrose, Lactose, Raffinose and Maltose, and does not ferment Mannose, Arabinose, Xylol and Menthol , as shown in Table (4-4), these results are consistent with what was reported in the (Bergeys Manual,2012).

Picture (4-1) Colonies of *B. bifidum* Growing on Solid MRS-L-Cystiene-HCL Medium

Table (4-2) Phenotypic Assays for Diagnosing *B. bifidum* Isolated from Different Sources

N	The Assay	Assay Result
1.	Gram Stain	+
2.	Growth Aerobic	-
3.	Growth Anerobic	+
4.	Morphology Rods	+

-: Negative Assay

+: Positive Assay

Table (4-3) Biochemical Assays for the Diagnosis of *B. bifidum* Isolated from Different Sources

N	The test	Test result
1.	Ammonia from arginine	-
2.	Catalase	-
3.	Citrate Utilization	-
4.	Gas production	-
5.	Gasein hydrolysis	-
6.	Gelatinase	-
7.	Growth temperature (35-45)	+
8.	Growth temperature (5-15)	-
9.	Indole	-
10.	KOH	-
11.	Lecethinase	-
12.	Lipase	-
13.	Motility test	-
14.	Nitrate Reduction	-
15.	Oxidase	-
16.	Starch hydrolysis	-
17.	Urease	-

-: Negative Assay

+: Positive Assay

Table (4-4) Fermentation of Sugars for the Diagnosis of *B. bifidum* Isolated from Different Sources

N	Sugar name	Test result
1.	Arabinose	-
2.	Fructose	+
3.	Galactose	+
4.	Glucose	+
5.	Lactose	+
6.	Maltose	+
7.	Mannose	-
8.	Manthol	-
9.	Raffinose	+
10.	Sucrose	+
11.	Xylol	-

-: Negative Assay

+: Positive Assay

4-2- Bacterial Diagnosis Using VITEK- 2 Compact System – Identification

To obtain a more accurate diagnosis of *B. bifidum* isolates, diagnostic Assaying was performed using the VITEK-2 device technology, it is one of the devices that identifies the types of microorganisms within a short period of time and accurately. The device was developed by the French company Biomerieux , automatically identifies the type of bacteria in the device by performing 64 Biochemical Assays without the need for any other Assays .

In the present study, a diagnostic card for Gram-positive anaerobic bacteria was used , its most important feature is that it recognizes living cells only using the identification card , the diagnostic level of the organism is determined by its Assay map and compared to the classification characteristics of the device , it gives the

organism a probability percentage in diagnosis. For example, if the probability percentage is 96-99%, then it is at an excellent confidence level (Mondelli *et al.*,2012), The diagnosis result in the VITEK-2 device showed that all isolates belonged to *B. bifidum* bacteria.

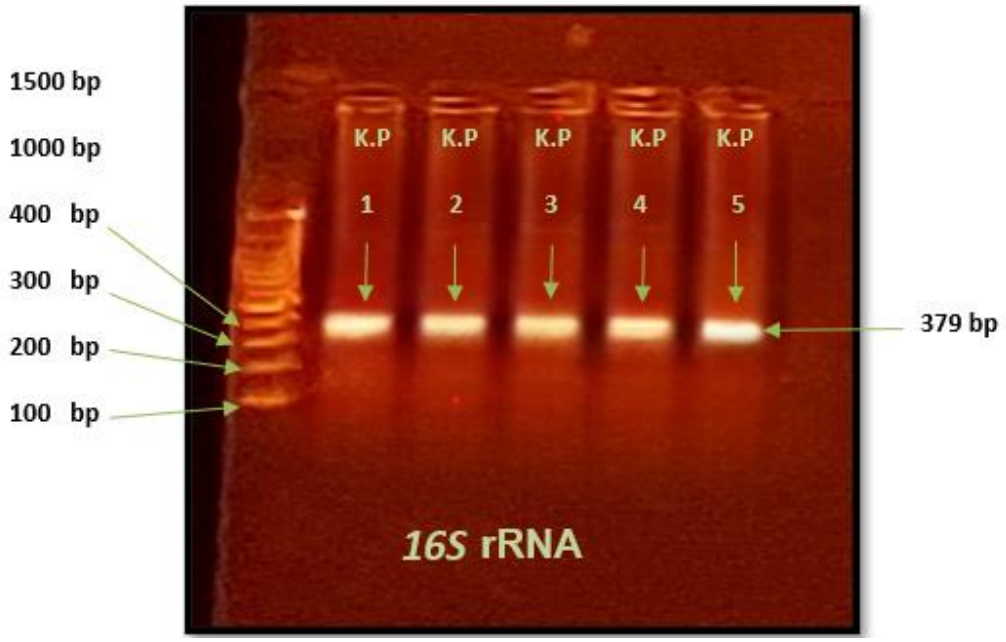
4-3- Molecular Diagnosis of *B. bifidum* Using (PCR)

Conventional diagnostic techniques are the main methods used in the diagnosis of bacterial isolates, although they take a long time , it requires many biochemical Assays and can often be expensive, especially when used to diagnose fastidous organisms, therefore, molecular diagnostic methods using Polymerase Chain Reaction technology are the best alternative as they are a rapid, Highly sensitive and specialised despite the high cost (Clifford *et al*., 2012).

In the current study, the *16S rRNA* gene was used to diagnose B. bifidum with a Pack size of 379, using Specialized qualitative prefixesfor this gene , this gene is characterized by high stability and unchangeable over time , therefore, it is a good criterion for diagnosis (Janda & Abbott *et al*.,2007).

The *16S rRNA* gene also contains highly variable regions. between bacterial species this will provide a specific sequence in each type of bacteria that will help in identifying the species (Elgaml *et al*.,2013; Biswas,2024).

The results showed that the five isolates belonged to *B.bifidum* bacteria, as shown in the picture (4-2), which shows that the primer specific to the gene (*16S rRNA*), which is (379 bp) in size, was effective in amplifying this gene.

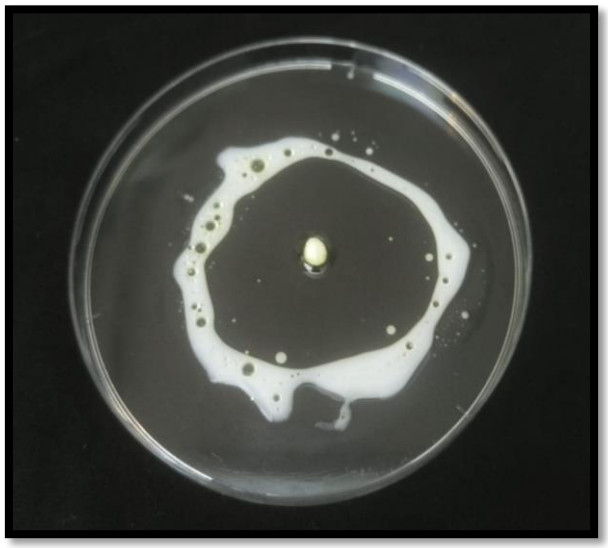


Picture (4-2) Electrophoresis of the PCR Product of *B. bifidum* Using the *16S rRNA* Gene-Specific Primer (379 bp), 1.5% gel Concentration and 70V Voltage, for 50 Minutes

4-4- Ability of *B. bifidum* to Produce Biosurfactant

The potential of bacteria to produce Biosurfactant (BS) was investigated by performing an oil spreding technique , after diagnosing and confirming the isolates, a positive result was shown in the ability of all bacterial isolates to displace oils, which is evidence of the bacteria's ability to produce a Biosurfactant dispersant as in picture (4-3).

A study conducted in India showed the ability of *Pseudomonas spp*. isolated from oil-saturated soil to produce Biosurfactant (Anandaraj & Thivakaran,2010), another study conducted in Spain Assayed the ability of Lactobacillus spp. bacteria to produce surface bio-dispersants (Rodrigues *et al*.,2006).



Picture (4-3) Ability of *B. bifidum* to Produce Biosurfactant (BS) by Oil Dispersion Assay

4-5- Emulsification index (E24%) of *B. bifidum* Isolates

The results of the Emulsification Index (E24%) test for *B. bifidum* isolates, the appearance of a foam layer after 24 hours of interaction of the bacterial culture with kerosene – indicate the ability of the bacteria to produce Biosurfactants of the glycolipids type, This indicates that the bacteria have the ability to emulsify fats, As in picture (4-4).

Picture (4-4) Emulsification Index (E24%) of *B. bifidum* Isolates in Kerosene

This ability is essential not only in evaluating the physical properties of a compound, such as reducing surface tension, but also in confirming the functional role of these compounds in biological applications, Isolates that achieved E24 positive results demonstrate their ability to stabilize water–oil emulsions, a behavior clearly indicative of efficient glycolipid production. Glycolipids are among the most important classes of low-molecular-weight biosurfactants known for their ability to reduce surface tension by up to 90% compared to the water–oil interface. (Rahman *et al*.,2010).

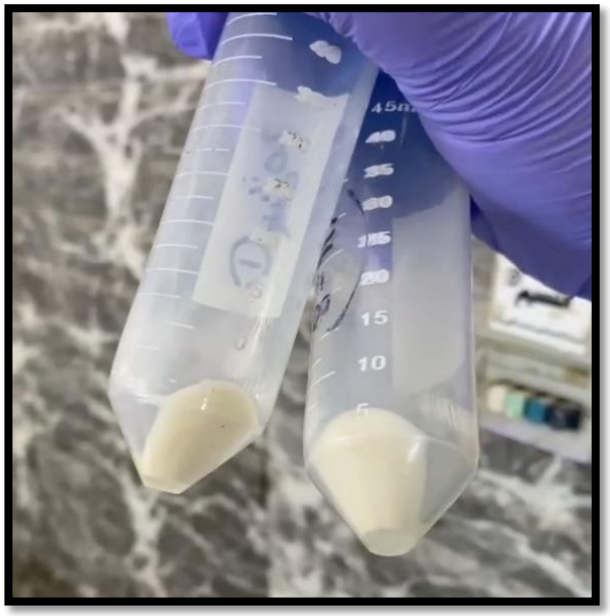
Recent studies have shown that Glycolipid Biosurfactants are effective in biosensing and also possess strong antibacterial and antifungal properties and reduce the formation of Biofilms by pathogenic microorganisms. This enhances their potential for use in Biomedicine, the success of *B.bifidum* isolates in forming self-emulsions is an indication of their stability and ability to cope with changing temperatures and environmental conditions (Huang *et al*.,2021).

Compared to other sources of Biosurfactants, Glycolipids are often extracted from fungi, *Pseudomonas*, and *Candida*, However, their production in human-based probiotic bacteria such as *B.bifidum* is promising, given their biocompatibility and bioavailability with the human body, Glycolipid Biosurfactants have anticancer properties and selective cytotoxicity. Studies have shown that some Glycolipids (such as Rhamnolipids and Sophorolipids) have a selective effect against cancer cells without significant harmful effects on normal cells, making them promising candidates as therapeutic or adjuvant agents for chemotherapy (Huang *et al*.,2021).

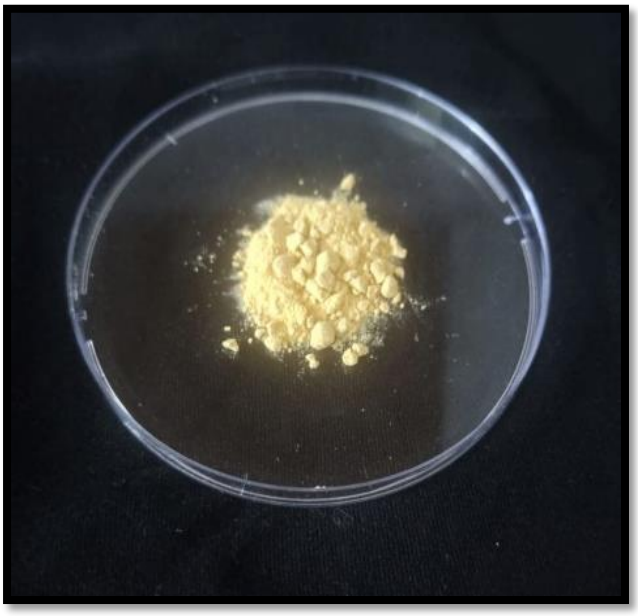
4-6- Biosurfactant (BS) Produced from *B. bifidum*

After extracting the Biosurfactant from the culture of *B. bifidum* anaerobically activated in MRS-L-Cysteine medium and after completing all extraction steps, the (BS) was collected by separating the filtrate from the sediment containing the

Biosurfactant dispersant as shown in picture (4-5), then it is dried with a Lyophilizer to obtain a white powder with a pale yellow color, as shown in picture (4-6).



Pictures (4-5) Precipitate of *B. bifidum* Bacteria



Pictures (4-6) Biosurfactant after Lyophilization

These results were close to the results of the study (AL-Mjalawi,2015) in which the Biosurfactant produced from *Bifidobacterium* spp. was extracted to inhibit the biofilm of pathogens isolated from cardiac catheterization patients and its effect on the phagocytosis process.

As shown by the results of another study in which the Biosurfactant Rhaminolipids were extracted from *Pseudomonas aeruginosa* bacteria (Salleh *et al*.,2011), another study showed that the probiotic bacteria Lactobacillus produces the Biosurfactant Glycosyldiglyceriodes, which exhibits antimicrobial activity and inhibits the adhesion of pathogens (Gudin *et al*.,2010).

Another study showed that Biosurfactants have high effectiveness in reducing the percentage of pathogenic bacteria without inhibiting cell growth through

changes that occur on the surface of the bacteria, represented by surface tension and the charge of the bacterial cell wall (Roderigues *et al*.,2006), Another study by (Walencka *et al*.,2008), confirmed that Biosurfactants play an twa done in amplifying the interaction between cells and different cell surfaces.

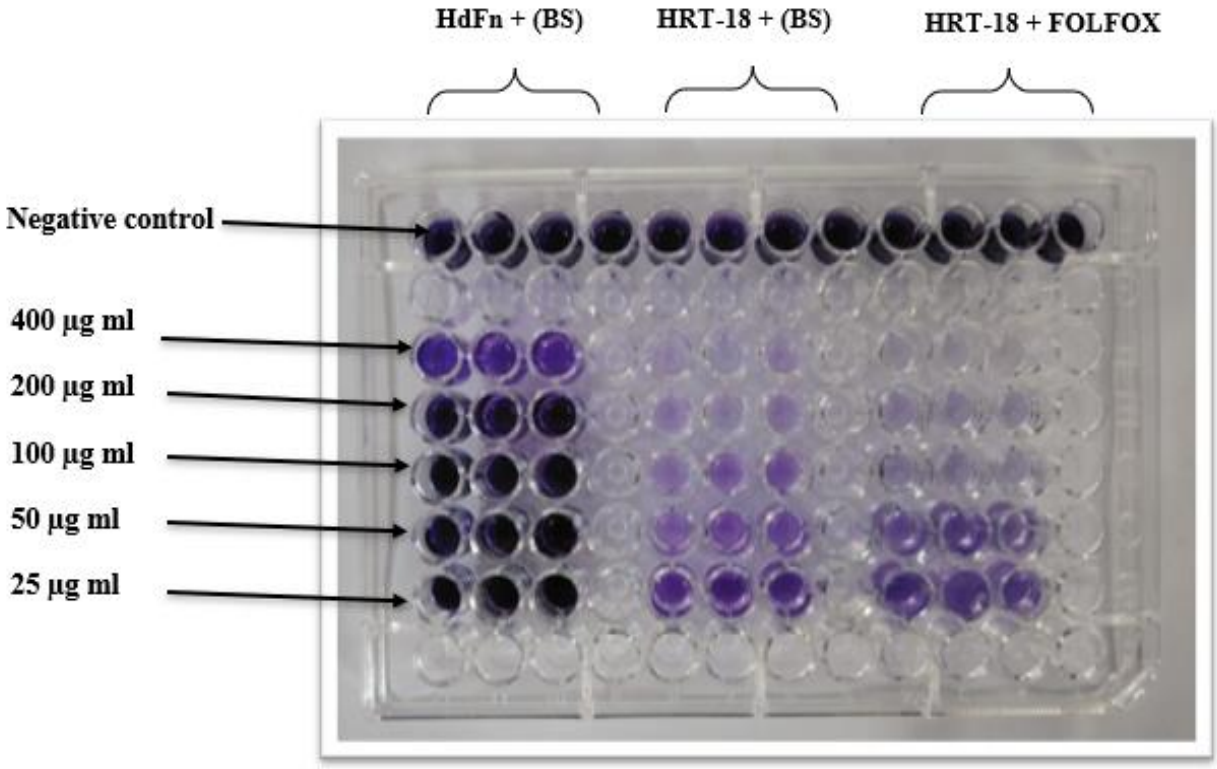
4-7- Detection of the Counteractive Activity of Biosurfactant in Colorectal Cancer cell Line [HRT-18]

The anti-Biosurfactant activity in [HRT-18] cell line was revealed by the following :

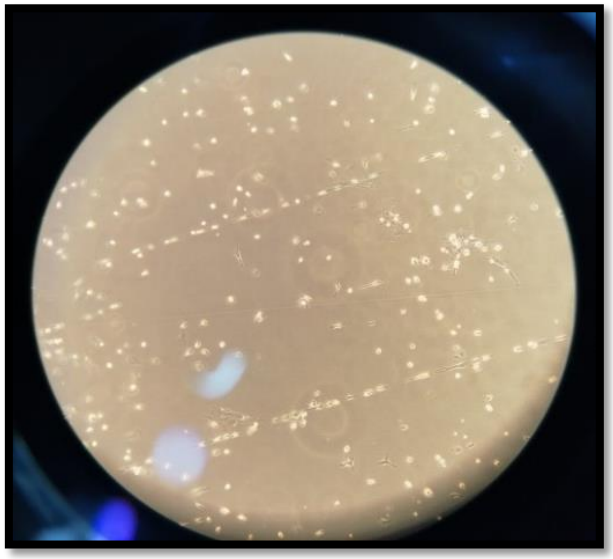
4-7-1- Cytotoxicity Results of Biosurfactant (BS)

Cytotoxicity assay MTT was used to investigate the toxic effect of Biosurfactant (BS) extracted from *B. bifidum* on [HRT-18] colorectal cancer cell line. This Assay is based on the conversion of MTT [3-(4-5dimethylthiazol-2yl)2,5-dipheny tetrazolium bromide] yellow by the enzyme succinate dehydrogenase (found in mitochondria) to the insoluble purple formazan Shown in picture (4-7) and pictures A , B (4-8), images showing [HRT-18] cell line , normal HDFn cells after treatment with Biosurfactant.

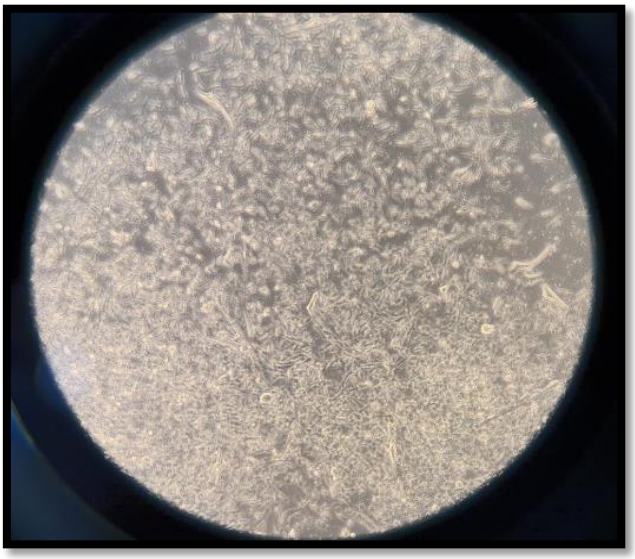
The Assay results showed that the Biosurfactant (BS) had good efficacy against the [HRT-18] colorectal cancer cell line, as it inhibited the cells by (68.4, 61.3, 48.3, 36, 24.4)%. The Biosurfactant (BS) did not show acute cytotoxicity on normal HDFn cells, as the inhibition rates ranged (19.6, 12.7, 6.7, 5.6, 4.7)% at concentrations (400, 200, 100, 50, 25) µg/ml, respectively, as shown in Table (4-5).



Picture (4-7) MTT Assay Plate of the Color Change to Purple for Formazan After Exposure of the [HRT-18] and [HDFn]Cell Line to different Concentrations of (BS), FOLFOX for 24 Hours at 37°C.



Picture (4-8) A [HRT-18] Cancer Cells Line After Treatment with Biosurfactant (BS)



Picture (4-8) B [HdFn] Normal Cell Line After Treatment with Biosurfactant (BS)

"At 40× magnification and a concentration of 200 µg/mL"

Table (4-5) Effect of Biosurfactant (BS) Extracted from *B.bifidum* on Colorectal Cancer Cell Line [HRT-18] and Normal Cell Line [HDFn] Using MTT Assay for 24 Hours Exposure Time at 37C°

(BS)			
Concen. µg ml	**Dead cell / Inhibition % (Mean±SD)**		***p* value**
	HDFn	**HRT-18**	
400	19.56±0.75	68.44±1.34	≤ 0.0001 **
200	12.73±0.9	61.35±1.63	≤ 0.0001 **
100	6.75±0.29	48.30±4.82	≤ 0.0001 **
50	5.63±0.60	36.00±2.78	≤ 0.0001 **
25	4.67±0.52	24.42±3.22	0.0005 **
** ***p* value ≤ 0.01**			

In addition, the results showed significant differences (P≤0.0001) in the calculation of IC50 when treating [HRT-18] cancer cells with the Biosurfactant (BS), which reached (53.4) µg/ml, the normal cell line HDFn reached (93.2) µg/ml, as shown in Figure (4-1).

Recent research has indicated that probiotics can affect the growth of cancer cells and reduce their ability to spread, although studies specifically focusing on the [HRT-18] cell line are still limited, findings from other studies suggest that *B. bifidum* may have inhibitory effects on tumor growth (Sankarapandian *et al*., 2022).

Several studies have also indicated the potential effects of *B. bifidum* probiotics and that certain strains of *Bifidobacterium* can boost the body's immune response,

which may help reduce tumours, for example these strains promote the production of interferon-γ, an important molecule in the immune response (Pei *et al*.,2024).

There is also evidence to suggest that *B. bifidum* can interact with chemotherapies and act synergistically increasing the effectiveness of treatments such as Oxaliplatin, this interaction may have a positive effect on [HRT-18] cells, enhancing the effectiveness of chemotherapy used to treat colon cancer (Lee *et al*.,2021).

Recent research has confirmed that *B. bifidum* may have positive effects on colon cancer cells, including enhancing the immune response and improving the efficacy of chemotherapy treatments. However, further studies are still needed to determine the exact mechanisms and how to apply these findings in clinical treatments (Faghfoori *et al*.,2021).

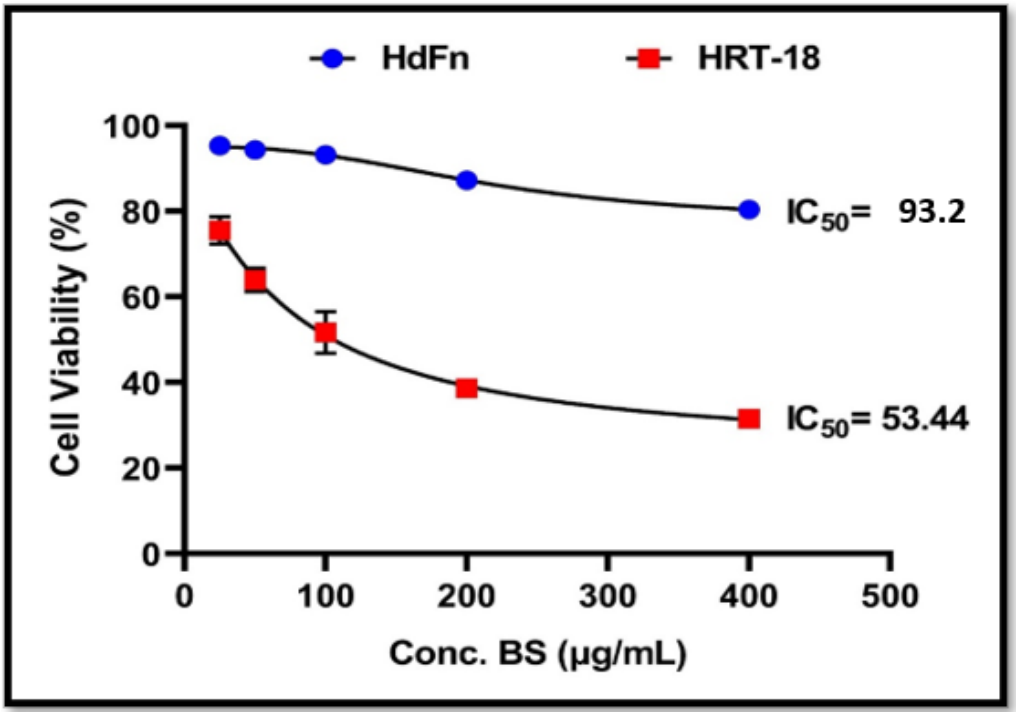


Figure (4-1) IC50 curve (mean±SD%) for the Colorectal Cancer Cell Line [HRT-18] and the normal cell line [HDFn] when treated with the (BS) extracted from *B. bifidum* using the MTT Assay at 37C°, 5% CO2 for 24 Hours.

4-7-2- Results of Cytotoxicity Assay of Chemotherapy (FOLFOX)

The results of the MTT cytotoxicity assay when the colorectal cancer cell line [HRT-18] and a normal cell line were treated with FOLFOX at different concentrations showed inhibition , the inhibition rates in the [HRT-18] cell line were (56.7, 48.8, 33.6, 19.6, 6.3)%, The inhibition rates in the normal cell line were (28, 17, 6.9, 5.9, 3.2)% at concentrations of (400, 200, 100, 50, 25) µg/ml , respectively is shown in Table (4-6). The results of calculating the IC50 for [HRT-18] cancer cells were (77.1) µg/ml and for the normal cell line [HDFn] (98.3) µg/ml, shown in Figure (4-2).

Table (4-6) Effect of FOLFOX on the Colorectal Cancer Cell Line [HRT-18] and the Normal Cell Line HDFn Using the MTT Assay at 37C°, 5% CO2 for 24 Hours

FOLFOX			
Concen. µg mL^{-1}	**Dead cell / Inhibition % (Mean±SD)**		***p* value**
	HdFn	**HRT-18**	
400	28.05±3.05	56.76±2.74	0.0003 **
200	17.09±0.57	48.81±2.59	≤0.0001 **
100	6.91±3.74	33.65±3.48	0.0008 **
50	5.94±1.38	19.65±1.64	0.0004 **
25	3.20±0.94	6.38±4.04	0.2553 NS
** ***p* value ≤ 0.01 , NS: Non-Significant**			

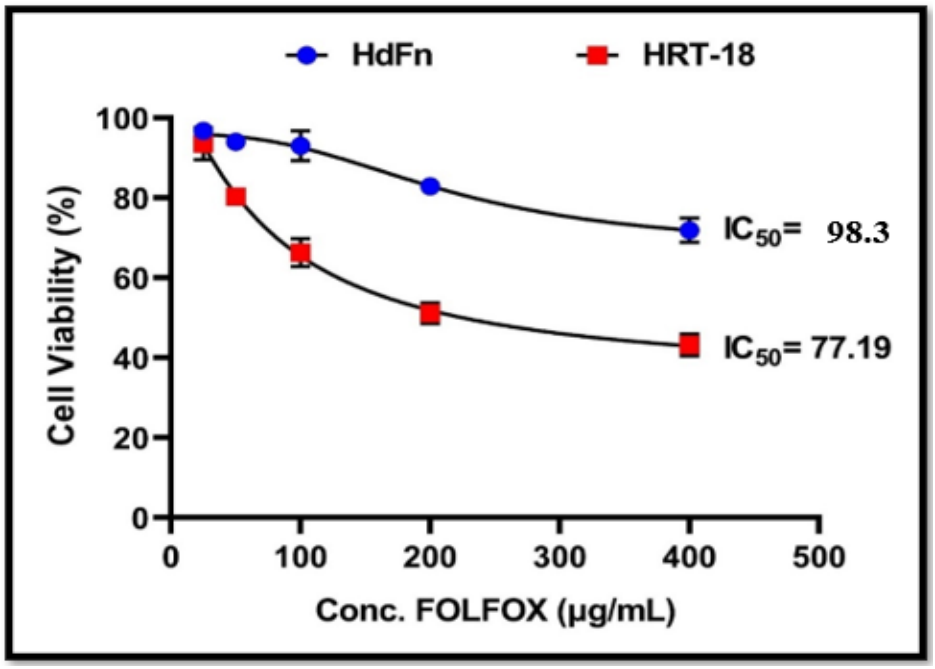


Figure (4-2) IC50 Curve (mean±SD%) for [HRT-18] Colorectal Cancer Cell Line and [HDFn] Normal Cell Line Treated with FOLFOX , Using MTT Assay at 37C°, 5% CO2 for 24 Hours

The results of calculating the IC50 for treating the cancer cell line [HRT-18] with (BS) showed that it reached 53.4µg/ml, which is lower compared to the chemotherapy FOLFOX used as an effective drug against colorectal cancer, which reached 77.1 µg/ml, and (BS) showed less killing effectiveness against normal HDFn cells compared to the chemotherapy FOLFOX.

FOLFOX is a combination chemotherapy consisting of 5-fluorouracil, folinic acid and oxaliplatin, also called oxaliplatin de Gramont or OxMdG, it is used to treat colorectal cancer. Researchers have studied the effect of FOLFOX on tumour and normal cell lines under specific conditions using the MTT assay to evaluate the effect of this treatment (Rothenberg *et al*.,2003).

FOLFOX can also have a significant inhibitory effect on colorectal cancer cell growth and the results of a study indicated a significant reduction in cell survival after exposure (Kowalewicz-Kulbat *et al*.,2023)

The results of another study showed that FOLFOX affects normal cell lines, however, the response may vary depending on the biological characteristics of each cell line, and it is important to note that this treatment may cause cytotoxicity affecting healthy cells, necessitating the development of strategies to mitigate these adverse effects (Zhou *et al*.,2022). FOLFOX is also thought to work by inhibiting DNA synthesis, leading to cancer cell death, as oxaliplatin interacts with cell DNA, causing a cellular response leading to programmed cell death (Park *et al*.,2005).

Clinical studies have shown its efficacy in improving survival rates, however, the development of treatment resistance calls for the search for new strategies to optimise the efficacy of FOLFOX (Narayan *et al*.,2022).

The current study indicated that FOLFOX has inhibitory effects against the [HRT-18] and [HDFn] cell line, with differences in response between the two lines, while BS has the same inhibitory effects on the [HRT-18] cancer line with significantly less toxic effects on the normal [HDFn] cell line. The MTT assay is an effective tool to assess these effects, providing valuable information for the development of new therapeutic strategies.

The results showed significant differences of $P \leq 0.0001$ between (BS) and FOLFOX in the HRT-18 cancer cell line at concentrations (400, 200, 100, 50, 25) µg/ml, shown in Figure (4-3).

The results showed significant differences between (BS) and FOLFOX in the normal HDFn cell line at concentrations of (400, 200) µg/ml and no significant differences at concentrations of (100, 50, 25) µg/ml as shown in Figure (4-4).

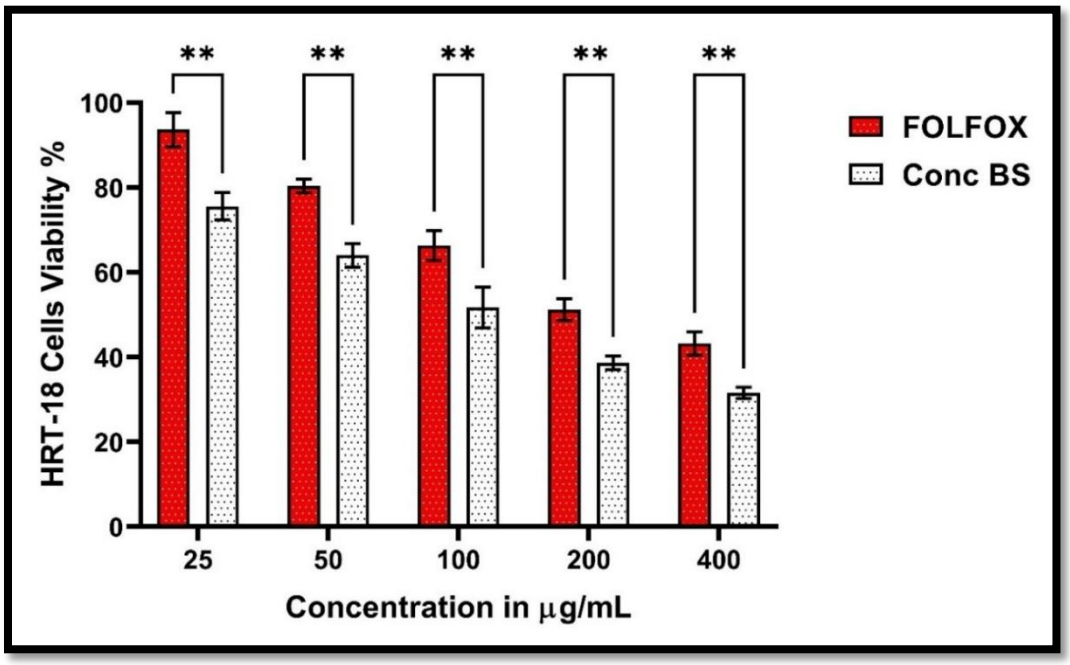


Figure (4-3) Comparison of the Effect of the Biosurfactant and the FOLFOX on the [HRT-18] Cell Line Using the MTT Assay at 37C°, 5% CO2 for 24 Hours

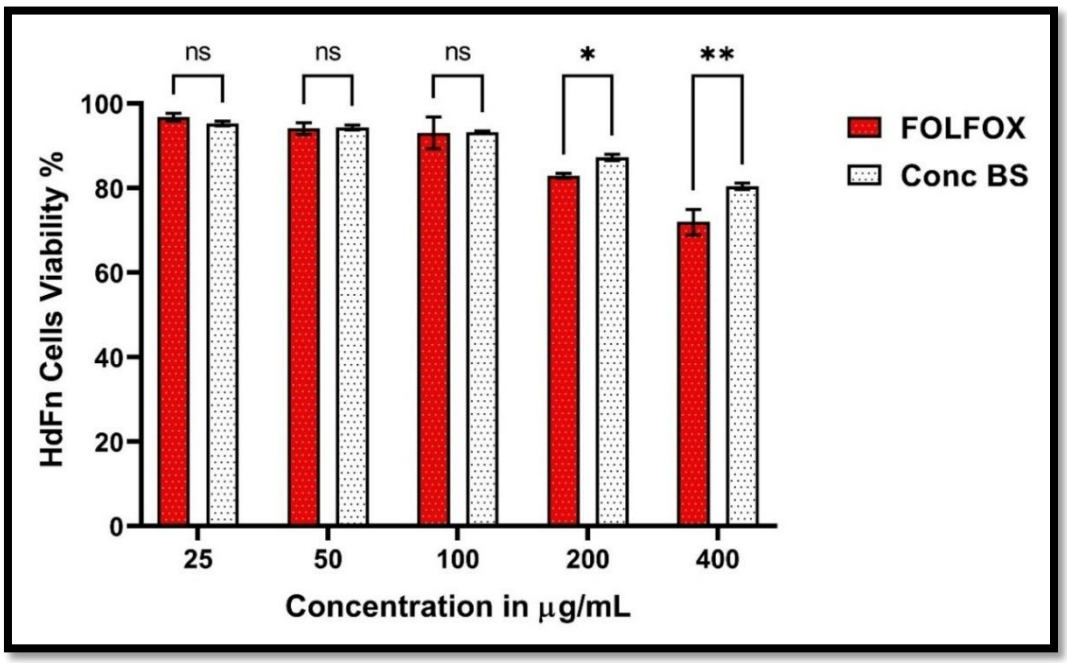


Figure (4-4) Comparison of the Effect of the Biosurfactant and the FOLFOX on the Normal [HDFn] Cell Line Using the MTT Assay at 37C°, 5% CO2 for 24 Hours

According to the Food and Drug Administration (FDA), the IC50 value represents the concentration of a drug required to inhibit 50% of in-vitro reactions, and is comparable to the EC50 value, which represents the concentration or dose effective in producing 50% of the maximum response to an inhibitory drug substance, the EC50 value also represents the concentration of serum or plasma required to achieve 50% of the effect in living cells.

The IC50 value is calculated in several ways, including using a curve fitting program, or through a point-to-point analysis that uses internal Excel templates. The raw data (relative fluorescent units or scintillation units) is plotted against the drug concentration, or using Excel and according to the basic equation for IC50 is IC50 = -log10 (x/C), where x is the concentration of the compound and C is the concentration of the inhibitor.

Several studies have confirmed the efficacy of probiotics in terms of their anti-cancer effects such as affecting pathogenic bacteria, inactivating carcinogenic compounds, especially those derived from food with different rates of inhibition depending on the concentration and type of cancer cell line (Saber *et al*.,2017). The results of another study showed the effectiveness of Probiotics in strengthening the gut barrier and reducing the risk of colorectal cancer (Kuugbee *et al*.,2016).

Other studies have confirmed the role of *Bifidobacterium* in inhibiting many pathogens due to its ability to lower the pH by producing lactic acid and acetic acid (Turroni *et al*.,2017). In the results of another study, it was indicated that some types of *Bifidobacterium* are able to reduce DNA damage caused by carcinogens, pre-tumor lesions, and tumors in the colon (Liboredo *et al*.,2013). It also confirmed the ability of *B. bifidum* and *B. infantum* to change the gut microbiota and reduce the development of colon cancer (Kuugbee *et al*.,2016).

A recent study investigated the effect of *Bifidobacterium breve*, *Lactobacillus reuteri*, and ten other Lactobacillus and *Bifidobacterium* strains on the human

colon adenocarcinoma cell line (LS174T) in a colorectal cancer model. This study demonstrated the potential of *Bifidobacteria* therapy due to their high cytotoxicity which causes apoptosis on LS174T cells compared to other bacterial therapies (Asadollahi *et al*.,2020).

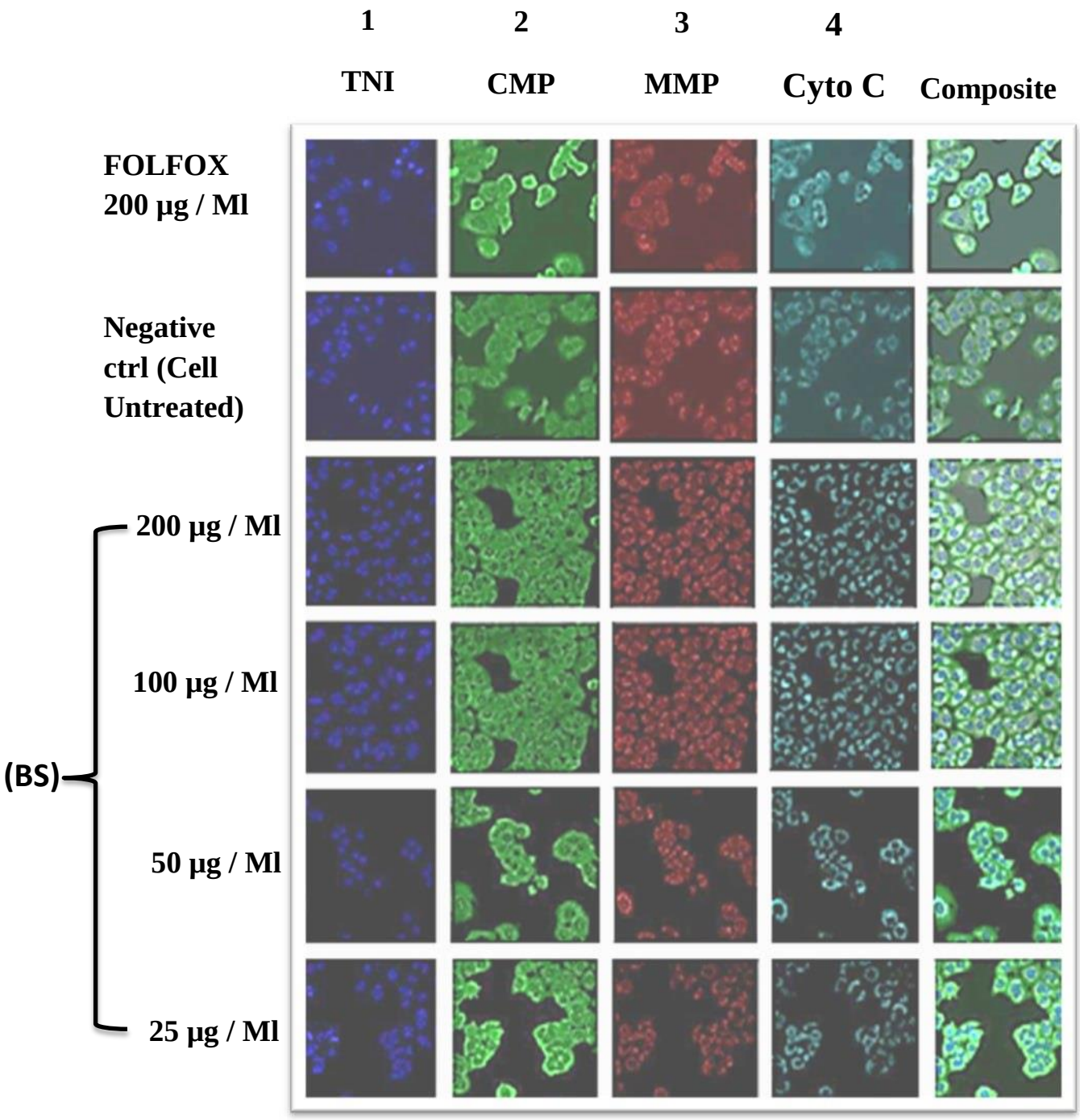
In another study, the anticancer effect of SPM0212 extract purified from *Bifidobacterium* adolescentis isolated from faecal samples of healthy people on human colon cancer cell lines (Caco-2, HT-29, and SW480) was reported and the results showed that the extract was significantly effective and inhibited the proliferation of these cancer cell (Lee *et al*.,2008).

4-7-3- High Content Screening (HCS)

This assay was used to measure additional cellular markers of the [HRT-18] colorectal cancer cell line and to confirm the efficacy of the Biosurfactant (BS) , including Viable Cell Count (VCC), Total Nuclear Intensity (TNI), Cell Membrane Permeability (CMP), Mitochondrial Membrane Potential (MMP) and Cytochrome C Releasing (CC), this is done by detecting the changes in the cells, as shown in picture (4-9). The Assay included several concentrations of bio-surfactant (BS) that showed effectiveness in the MTT Assay, namely (200, 100, 50, 25)µg /ml.

All treatments were compared with the negative control (Untreated) and the positive control FOLFOX at a concentration of 200 µg /ml.The results of the (HCS) examination shown in Table (4-7) A,B showed the following.

The study documented the efficacy of (BS) on the [HRT-18] cell line, resulting in a significant decrease in the number of live cancer cells, an increase in nuclear staining intensity, higher membrane permeability, a decrease in mitochondrial potential, and an increase in cytochrome C releasing, the resulting biochemical indicators, namely a decrease in MMP, indicate mitochondrial dysfunction and the onset of programmed cell death.



Picture (4-9) Multi-Indicator Cytotoxicity (HCS) Analysis of [HRT-18] Cells Treated with Different Concentrations of (BS) After 24 Hours of Incubation at 37C°

Channel 1: Hoechst Stain\ Channel
2: Cell Membrane Permeability Staining\ Channel
3: Mitochondrial Membrane Potential Staining Channel
4: Cytochrome C Staining

Table (4-7) A Multi-parameter Cytotoxicity (HCS) Analysis of [HRT-18] Cells Treated with (BS) Extract from *B. bifidum* at Different Concentrations After 24 Hours of Incubation at 37C°

Parameters	Mean ± SD Fluorescent Intensity (RFU)%						Sig.	*p* Value	LSD (F Value)
	Control	FOLFOX 200 µg / mL	BS Concentration in µg / mL						
			200	100	50	25			
VCC	4588± 77.1	2656± 292.7	2596± 68.6	3181± 8.0	3991± 332.1	4593± 72.12	**	≤0.0001	47.42
TNI	477.5± 3.6	575.5± 41.7	652.5± 4.9	548.0± 7.1a	484.5± 14.9	470.5± 9.2	**	0.00004	28.85
CMP	153.0± 2.8	220.5± 17.7	224.5± 14.9	152.5± 16.3	156.5± 12.02	155.0± 4.2	**	0.00023	15.44
MMP	748.0± 10.0	500.0± 14.14	431.0± 5.7	617.0± 7.07	747.5± 9.2	747.5± 1.7	**	≤0.0001	56.8
Cyto C	443.5± 2.1	654.0± 46.7	640.0± 22.6	540.5± 31.8	446.0± 1.4	453.0± 15.6	**	0.00004	29.19
** $p \leq 0.01$,									

Table (4-7) B Fold of change

Parameters	Fold of Change					
	Control	FOLFOX	(BS) Concentration in µg / Ml			
			200	100	50	25
VCC	1.0	0.58	0.57	0.69	0.87	1.00
TNI	1.0	1.21	1.37	1.15	1.01	0.99
CMP	1.0	1.44	1.47	1.00	1.02	1.01
MMP	1.0	0.67	0.58	0.82	1.00	1.00
Cyto c	1.0	1.47	1.44	1.22	1.01	1.02

The First Indicator: Viable Cell Count (VCC) Estimation

The results of cell viability estimation confirmed the findings of the MTT Assay, showing a significant decrease $p \leq 0.0001$ in the viability of HRT-18 cells at concentrations of (200, 100)µg/ml , with reductions of (57, 69)%, respectively , meanwhile no significant difference was observed at concentrations of (50, 25)µg/ml where cell viability was (87, 100)% , respectively, compared to the negative control (Untreated cells). The results showed no significant difference between the effect (BS) at a concentration of 200µg/ml and the FOLFOX at the same concentration, as shown in Figure (4-5).

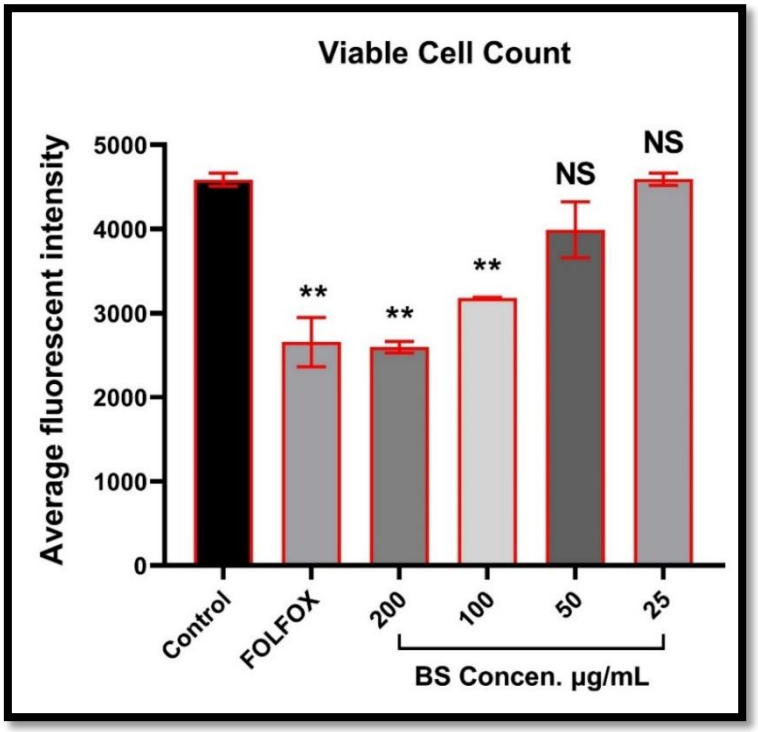


Figure (4-5) Effect of Different Concentrations of Biosurfactant (BS) Extracted from *B. bifidum* and FOLFOX on the (VCC) of [HRT-18] Cells Using the HCS Assay After 24 Hours of Incubation at a Temperature of 37C°

The MTT assay measures the metabolic activity of cells, while VCC measures the number of cells capable of surviving and proliferating, the MTT assay measures the metabolic activity of cells, while VCC measures the number of cells capable of surviving and proliferating, the count (VCC) results confirm the MTT assay results, indicating a significant decrease in the viability of [HRT-18] cells at concentrations of 100 and 200µg/ml. In contrast, no significant difference in cell viability was observed at concentrations of 25 and 50µg/ml compared to untreated cells, which enhances the reliability of the results, In addition, there was no significant difference between the effects of (BS) and FOLFOX at a concentration of 200 µg/ml.

The significant decrease in cell viability at high concentrations of 200 and 100µg/ml indicates that (B) may have a toxic effect on the [HRT-18] cell line. This effect could be caused by different mechanisms, such as inducing apoptosis or necrosis , the lack of significant effect on cell viability at low concentrations 50 and 25µg/ml suggests that these concentrations may be safe or ineffective; these concentrations may be insufficient to cause significant toxic effects on cells, the lack of significant difference between the effects of (BS) and FOLFOX suggests that they may have similar efficacy under the tested conditions for the treatment of colorectal cancer (Louis&Siagel,2011).

The Second Indicator: Total Nuclear Intensity (TNI)

The examination of morphological changes in HRT-18 colon and rectal cancer cells was based on staining the cells with Hoechst stain , the results of (TNI) showed a significant increase when treated with the (BS) at concentrations of (200, 100)µg/ml compared to the negative control (Untreated cells), with an increase of (37, 15)% respectively, as for concentrations (50, 25)µg/ml no significant differences were shown in the increase in (TNI) , the results showed no significant difference between the effect of (BS) at a concentration of 200µg/ml

and FOLFOX at a concentration of 200µg/ml on the Total Nuclear Intensity shown in Figure (4-6).

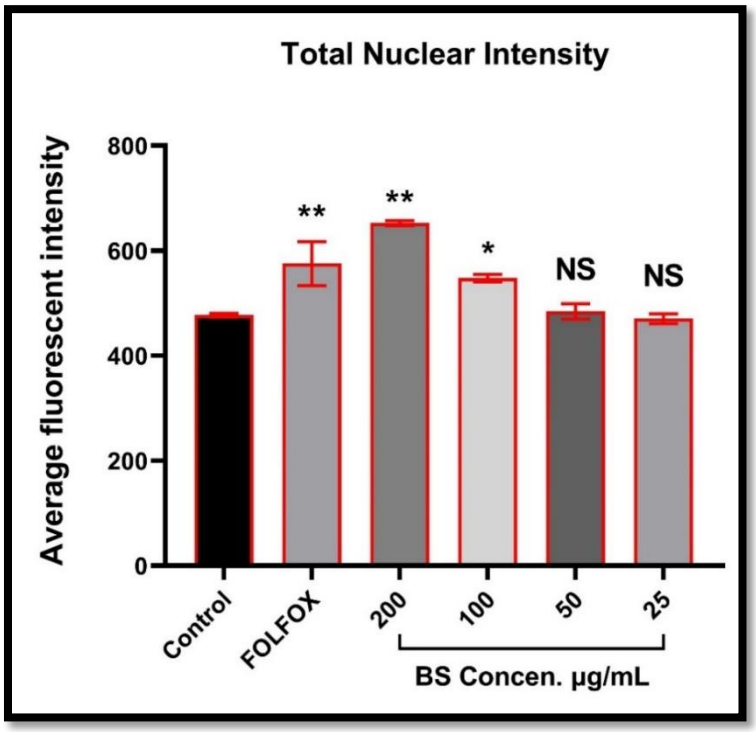


Figure (4-6) Effect of Different Concentrations of Biosurfactant (BS) Extracted from *B. bifidum* and FOLFOX on the (TNI) of [HRT-18] Cells Using the HCS Assay After 24 Hours of Incubation at 37C°

The results showed that treatment with (BS) at concentrations of 200 and 100 µg/ml resulted in a significant increase in TNI compared to untreated cells (negative control). this increase in nuclear fluorescence intensity indicates chromatin condensation or the onset of apoptosis, a sign of the cytotoxic effect of (BS) on [HRT-18] colorectal cancer cells.

In contrast, lower concentrations 50 and 25µg/ml showed no significant change, indicating that the effect of (BS) is dose-dependent, which is common in research on anti-cancer agents (Miyake *et al*.,2020). Furthermore, the lack of a significant difference between the effect of (BS) at a concentration of 200µg/ml and that of

FOLFOX at the same concentration reinforces the effectiveness of (BS) as an alternative or adjunct to conventional chemotherapy.

The current study supports recent studies showing that some Biosurfactant glycolipids have the ability to induce apoptosis through multiple mechanisms, including increased cell membrane permeability and effects on nuclear integrity, as reflected in measurements such as TNI, Nuclear morphology is a characteristic feature of programmed cell death (Hsu *et al*.,2025).

The Third Indicator: Cell Membrane Permeability (CMP)

The results showed significant differences for the (BS) at a concentration of 200 µg/ml, with a percentage of 47%, however, at concentrations (100, 50, 25) µg/ml no significant differences were shown compared to the negative control (untreated cells). The results indicated no significant difference between the effects of the (BS) at a concentration of 200 µg/ml and the FOLFOX at the same concentration, both resulting of 44%, as shown in Figure (4-7).

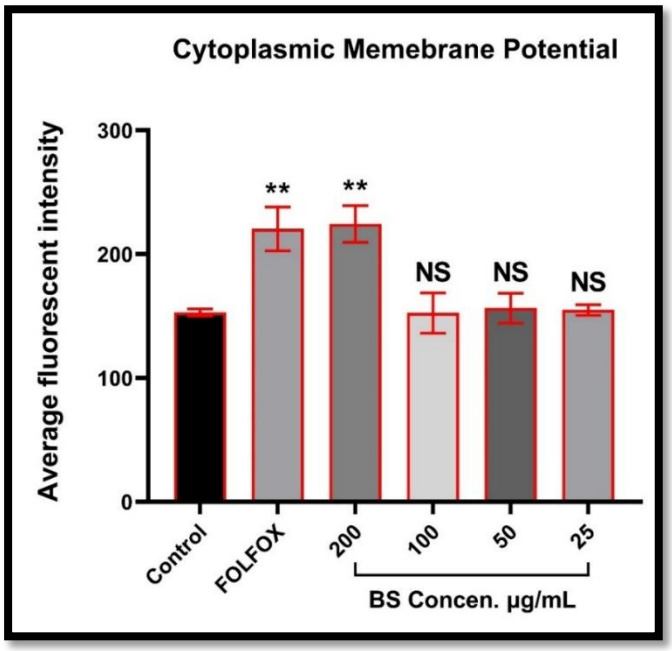


Figure (4-7) Effect of Different concentrations of Biosurfactant (BS) extracted from *B. bifidum* and FOLFOX on the (CMP) of [HRT-18] cell membrane using the HCS Assay after 24 hours of incubation at 37C°

The results indicate that (BS) significantly affects cell membrane permeability at a concentration of 200µg/ml. However, no significant effects were observed at lower concentrations 100, 50 and 25µg/ml compared to the untreated control, in addition, the effect of (BS) at 200µg/ml was similar to that of FOLFOX at the same concentration.

The observation indicates that (BS) has a significant effect at high concentrations with a dose-dependent response, this is consistent with many biological and pharmacological phenomena where a threshold concentration must be reached to produce an effect the reason may be saturation of binding sites on the cell membrane or the need for a higher concentration of (BS) to disrupt the membrane structure.

(BS) may cause temporary holes in the membrane, facilitating the passage of molecules that are normally restricted or (BS) may bind to membrane proteins and affect their function impairing membrane integrity Cell membrane because permeability is an indicator of its integrity (Rems *et al*.,2019).

The lack of significant difference between the effects of (BS) and FOLFOX at a concentration of 200µg/ml indicates that they have similar efficacy in altering membrane permeability.

The Fourth Indicator: Mitochondrial Membrane Potential (MMP)

Membrane strength is a hallmark of mitochondrial integrity, and membrane depolarization is an indicator of mitochondrial dysfunction, which is increasingly affected by drug toxicity and is an effective characterization of cell death signals as the efficiency of MMP decreases, cytokines present on the cell wall, including cytochrome C, is be released, that is as the MMP decreases, the CC mentioned as the fifth indicator in the HCS Assay increases (Zorova *et al*., 2017).

The results showed that the concentrations of the Biosurfactant (100, 200) µg/ml caused a reduction in the (MMP) by (58, 82)%, respectively , the results of

this indicator showed no significant differences in the concentrations (50, 25) µg/ml compared to the negative control (untreated cells) . The results showed no significant difference between the effect of the (BS) at a concentration of (200) µg/ml and the FOLFOX at a concentration of 200µg/ml, which amounted to (67)% on the s Mitochondrial Membrane Potential shown in Figure (4-8).

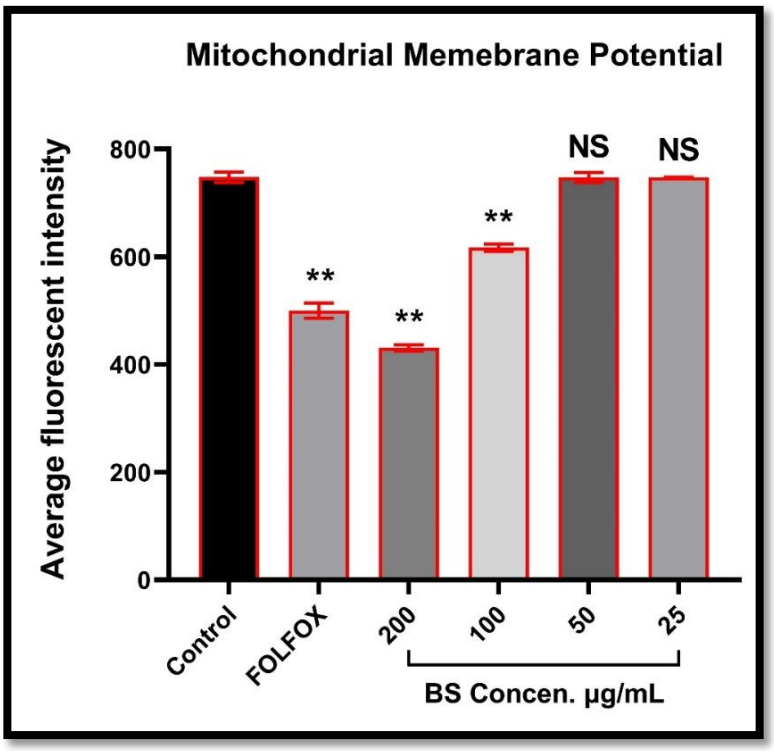


Figure (4-8) Effect of Different Concentrations of Biosurfactant (BS) Extracted from *B. bifidum* and FOLFOX on the (MMP) of [HRT-18] Cells Using the HCS Assay After 24 Hours of Incubation at a Temperature of 37C°

In this study, the use of the biosurfactant (BS) at concentrations of 100 and 200 µg/ml led to a significant decrease in MMP. This sharp decrease indicates that the substance caused a disruption in mitochondrial function, which may activate the intrinsic apoptosis pathway. This conclusion is supported by the increase in cytochrome C secretion another indicator studied in the same experiment, when MMP decreased, indicating the breakdown of the inner mitochondrial membrane.

It is noteworthy that the effect of biomaterial (BS) at a concentration of 200 µg/ml was almost similar to that of FOLFOX at the same concentration , which reinforces the possibility of using these natural compounds as alternatives or adjuvants to traditional chemotherapy treatments, especially since they may exhibit selective toxicity towards cancer cells without strongly affecting normal cells, as shown in some recent studies.

At the lower concentrations 25 and 50 µg/ml, there was no significant effect on mitochondrial membrane potential, indicating a dose-dependent effect, which is an important feature in assessing the efficacy and toxicity of compounds in preclinical studies (Li *et al*,.2022).

The Fifth Indicator: Cytochrome C Releasing (CC)

The results showed a significant increase in the amount of cytochrome C released from mitochondria when treated with (BS) at concentrations of (100, 200) µg/ml by (44, 22)%, respectively , no significant differences were found at concentrations of (25, 50) µg/ml compared to the negative control , the results showed no significant difference between the effect of the (BS) at a concentration of (200) µg/ml and the FOLFOX at a concentration of (200)µg/ml which increased by (47)% over the negative control, as shown in Figure (4-9).

The current study indicates that (BS) stimulates Cyt C release in a dose-dependent manner, with high concentrations 100 and 200 µg/ml leading to a marked increase in cytochrome C release mechanisms, the effect of (BS) on Cyt C release at a concentration of 200µg/ml is similar to that of FOLOX, which acts by damaging DNA and inhibiting cell division, leading to cell cycle arrest and cell death. Oxaliplatin is a major component of FOLFOX, stimulating DNA cross-links.

Cytochrome C is a therapeutic target due to its role in cell death. Cyt c has been explored as an anti-cancer agent some studies are investigating the delivery of

exogenous Cyt c to cancer cells to induce cell death, particularly in cancers where cell death pathways are impaired, blood levels of Cyt c could serve as a marker to assess the efficacy of cancer therapies (José *et al*.,2001).

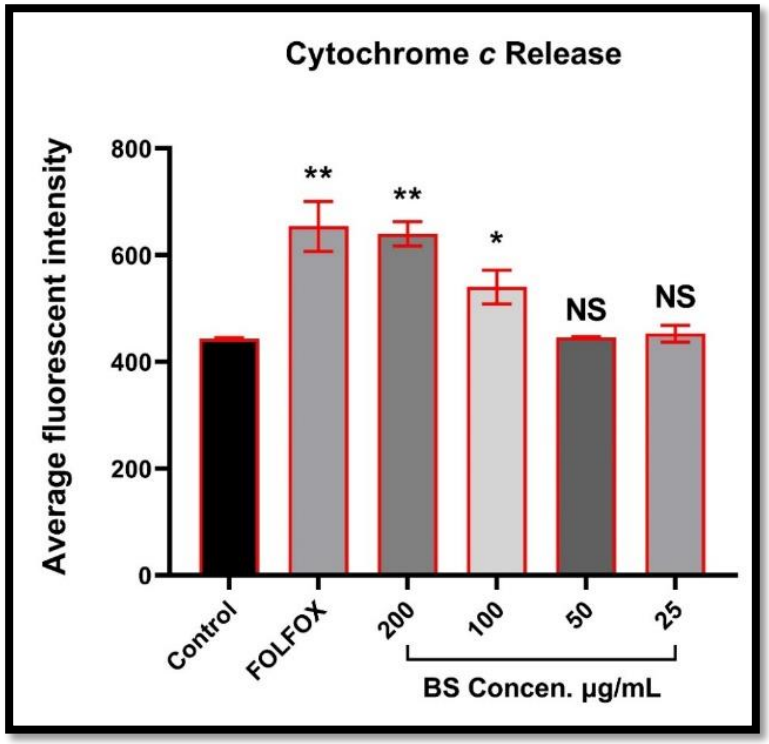


Figure (4-9) The Effect of Different Concentrations of Biosurfactant (BS) Extracted from *B. bifidum* and FOLFOX on the Release of Cytochrome C from [HRT-18] Cells Using the HCS Assay After 24 Hours of Incubation at 37C°

In the current study, High Content Screening (HCS) was used, also known as High Content Analysis or cellomics, this is an advanced technique used in biological research and drug discovery to analyze the effects of chemical compounds, genetic materials, or other biologically active molecules on cells. It aims to modify the phenotypic characteristics of the cells in a desired manner and investigate the resulting final phenotype of the cells. It also provides a more detailed and multifaceted analysis of cellular responses (Lin *et al*.,2020).

This assay included the measurement and monitoring of changes in Viable Cell Count, Total Nuclear Intensity, Cell Membrane Permeability, Mitochondrial Membrane Potential and Cytochrome C, as reported by (Vivek,2008), and

comparing them with untreated cells and the efficacy of the treatment. Thus, it holds particular value in complex and multifactorial diseases, such as neurological disorders or cancers in general (Li *et al*.,2015).

The results of the cytotoxicity evaluation of the studied indicators after treating them with different concentrations of the Biosurfactant (BS) extracted from *B. bifidum*. In addition the FOLFOX chemotherapy was introduced at a concentration of 200 µg/ml as a positive control, being an effective treatment against colon and rectal cancer, along with the necessary stains, and compared to the negative control of untreated cells after incubation at 37C° for 24 hours , observed Significant effects on most cellular indicators with increasing concentrations of (BS) in the [HRT-18] cell line, particularly at concentrations of (200,100)µg/ml, when compared to the negative control and in comparison to the FOLFOX at a concentration of 200µg/ml, The Biosurfactant (BS) at a concentration of 200 µg/ml did not show significant differences at $p \leq 0.05$. this clearly indicates that (BS) could be a promising treatment for colorectal cancer after successful clinical Assays at appropriate doses. this is attributed to programmed cell death by the effect of cytotoxicity.

The current study found clear effects on cellular markers that induce cells to enter the apoptosis process. One of the most important features of the early stages of programmed cell death is the disruption of mitochondrial activity, changes in the permeability of their membranes, and the redox system within them, which leads to the opening of membrane pores and allows the passage of ions and small molecules across the membrane, which leads to an imbalance in the ionic balance, which in turn leads to the separation of the respiratory chain and the release of Cytochrome C inside the cell (Elkholi *et al*.,2014).

The results of the current study were similar to the results of another study aimed at improving drug options for patients with colorectal cancer because these

patients are the first to receive targeted therapies that aim to prevent cancer-causing proteins from growing out of control while preserving as much healthy tissue as possible. However, some patients are unresponsive to these treatments because they have cancer-inducing mutations, which are believed to be the main reason for resistance to these treatments (Thomas *et al*.,2019), as many colorectal cancer patients who have used all known treatment options but are still suffering from the progression of their cancer are in need of new treatments.

Although FOLFOX is an effective chemotherapy that kills harmful cancer cells, it may damage normal cells and healthy tissues and cause a range of side effects , of every 100 people who receive FOLFOX, 3 or more may experience a heart attack or heart failure, which can cause shortness of breath, ankle swelling, fatigue, and brain damage, and posterior reversible encephalopathy syndrome, which can cause headaches, seizures, and blindness (Pucciarelli *et al*.,2006).

4-7-4- Assay Results Caspase-9 and Caspase-8

The results showed a significant increase in Caspase 9 protein compared to Control and this increase was dependent on the concentrations, when using different concentrations of (BS) ranging between (200, 25)µg/ml which reached between (4.16, 1.1)%, respectively while Caspase 8 showed lower activity of (1.88, 0.93)% compared to Caspase 9 indicating that the main pathway in the programmed death process is the intrinsic pathway, which is responsible for Caspase 9.

The results showed no significant differences between the effect of the Biosurfactant and FOLFOX at a concentration of 200µg/ml compared to the negative control (untreated cells) in both pathways, as the increase reached (4.16, 3.55)% in Caspase9 and reached (1.88, 1.67)% in Caspase8, as shown in Table (4-8)A,B .

Table (4-8)A Results of Caspase-9 and Caspase-8 Assays for [HRT-18] Cells Treated with (BS) Extracted from *B. bifidum* at Different Concentrations

Parameters	Mean ± SD Fluorescent Intensity (RFU)%						Sig.	*p* Value	LSD (F Value)
	Control	FOLFOX	BS Concentration in µg / mL						
			200	100	50	25			
Caspase 9	18196 ±782.8	64657 ±3852	75664 ±2796	53344 ±1008	43960 ±1846	19589 ±1492	**	<0.0001	221.1
Caspase 8	18142 ±677.4	30256 ±728.3	33973 ±969.4	28010 ±6018	25140 ±2266	16862 ±598.9	**	0.0039	12.62
** $p < 0.01$									

Table (4-8)B Fold of Change

Parameters	Fold of Change					
	Control	FOLFOX	BS Concentration in µg / mL			
			200	100	50	25
Caspase 9	1.0	3.55	4.16	2.93	2.42	1.08
Caspase 8	1.0	1.67	1.88	1.54	1.39	0.93

The results show an increase in Caspase-9 activity at (BS) doses from 25 to 200µg/ml, with a clear dose-dependent variation, a clear indication of effective concentration-dependent binding, The low Caspase-8 activity indicates that the extrinsic pathway is secondary, in comparison, Caspase-8 activation was

relatively lower, which reinforces that (BS) acts primarily through the intrinsic pathway, rather than through the extrinsic pathway.

It is noteworthy that (BS) at 200 µg/ml stimulated Caspase-9 by 4.16% compared to 3.55% for FOLFOX, while Caspase-8 was stimulated by 1.88% compared to1.67% a clear indication of its competitiveness with chemotherapy in triggering death pathways regardless of the method. In a similar study, it was shown that lipopeptide biosurfactant activates Caspase-9 and releases Cytochrome-C through its effect on mitochondrial membrane potential (MMP) (Li *et al*.,2022).

The mechanism of apoptosis occurs through two main pathways: the intrinsic pathway, which is centered around mitochondria and is considered the starting point for apoptosis. It receives several signals, including DNA damage, oxygen deficiency, and oxidative stress, which in turn lead to the production of cytochrome C in the mitochondrial membrane and the activation of proteins that aid in the process of apoptosis, followed by a series of reactions that lead to the formation of a protein called apoptosome, it works to break down and activate several enzymes that are effective in protein degradation (Mustafa *et al*.,2024).

While the extrinsic pathway relies on tumor necrosis factor receptors to stimulate cell death, these receptors bind to their counterparts inside the cells, they will activate Caspase 9 and 10, forming a complex that serves as a signal to initiate apoptosis. Westaby *et al*.,2022).

Caspases are enzymes (Cysteine aspartic protease) that belong to a family of enzymes playing a crucial role in apoptosis, they achieve this by directly activating caspases or through the stimulation of caspases in response to immune responses or inflammatory processes (Arcy,2019).

Researcher study (Abraham,2012), indicated that all eukaryotic cells are subject to the programmed cell death mechanism, which can be stimulated by stimuli from

inside or outside the cell, with a system subject to balance between cell death on the one hand and cell multiplication and cell differentiation on the other hand, any imbalance in this system leads to the activation of many diseases, including cancer (Arcy, 2019).

4-7-5- Assay Results of Anti-oxidant Activity (DPPH)

This Assay was conducted to determine the extent of oxidation (scavenging of free radicals) when treating the [HRT-18] cancer cell line with different concentrations of Biosurfactant (BS),

The results of this test showed no significant difference between the biosurfactant (BS) and ascorbic acid when treated the [HRT-18], with a slight difference at a concentration of 50µg/ml, the percentages in the cells treated with Ascorbic acid were (76.5, 72.5, 64.1, 52)%, while the percentages in the cells treated with (BS) were (73.6, 63, 51.7, 43.2)% at concentrations of (200, 100, 50, 25)µg/ml respectively as shown in Table (4-9) and Figure (4-10).

Table (4-9) DPPH Analysis of [HRT-18] cells Treated with Different Concentrations of (BS) that Extract from *B. bifidum* and Ascorbic Acid After 24 Hours of Incubation at 37C°

Concen. In µg/Ml	Mean ± SD Scavenging Activity (%)		Sig.	*p* Value
	Ascorbic Acid	BS		
200	76.51 ± 2.43	73.69 ± 0.74	NS	0.9378
100	72.53 ± 0.99	63.08 ± 1.34	NS	0.1305
50	64.12 ± 1.10	51.77 ± 2.49	*	0.0321
25	52.01 ± 1.87	43.24 ± 1.22	NS	0.1780

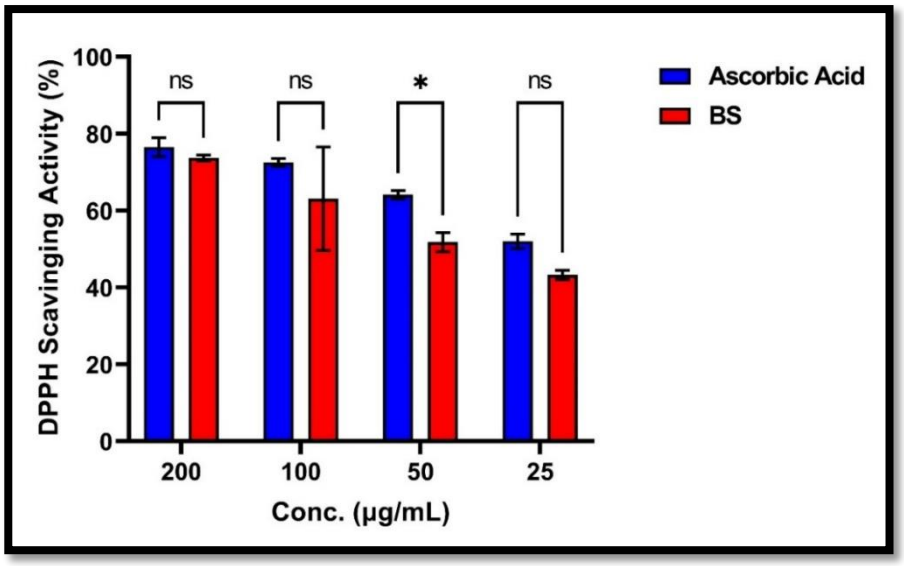


Figure (4-10) Comparison of the Effect of the (BS) Extracted from *B. bifidum* and Ascorbic acid on the [HRT-18] Cell Line After 24 Hours of Incubation at 37C°

DPPH is Assay of great importance in the fields of food, medicine, and biotechnology, and is used to develop and introduce new antioxidants (Flieger & Flieger,2020). To evaluate the antioxidant capacity by the DPPH method, the result of this process is based on the appearance of a color in the environment, the intensity of which can be measured using a spectrophoto meter , considered DPPH is a stable free radical that contains an unpaired electron on one of the nitrogen bridge atoms (Zeynep *et al*.,2017; Sharma&Bhat,2009).

The radical scavenging of DPPH is the basis for evaluating the antioxidant capacity as in the following equation:

DPPH (purple) + H – A → DPPHH (yellow) + A

The results of the DPPH free radical Assay in the current study were similar to many studies that have focused on probiotics as living microorganisms that have a beneficial effect on health by showing quantitative and qualitative effects on inAssayinal bacteria or modifying the functioning of the immune system , emphasised that there are several probiotic strains that have shown numerous clinically proven health benefits in recent years , the number of studies on the

antioxidant properties of probiotics has also increased significantly (Arkadiusz *et al*.,2021).

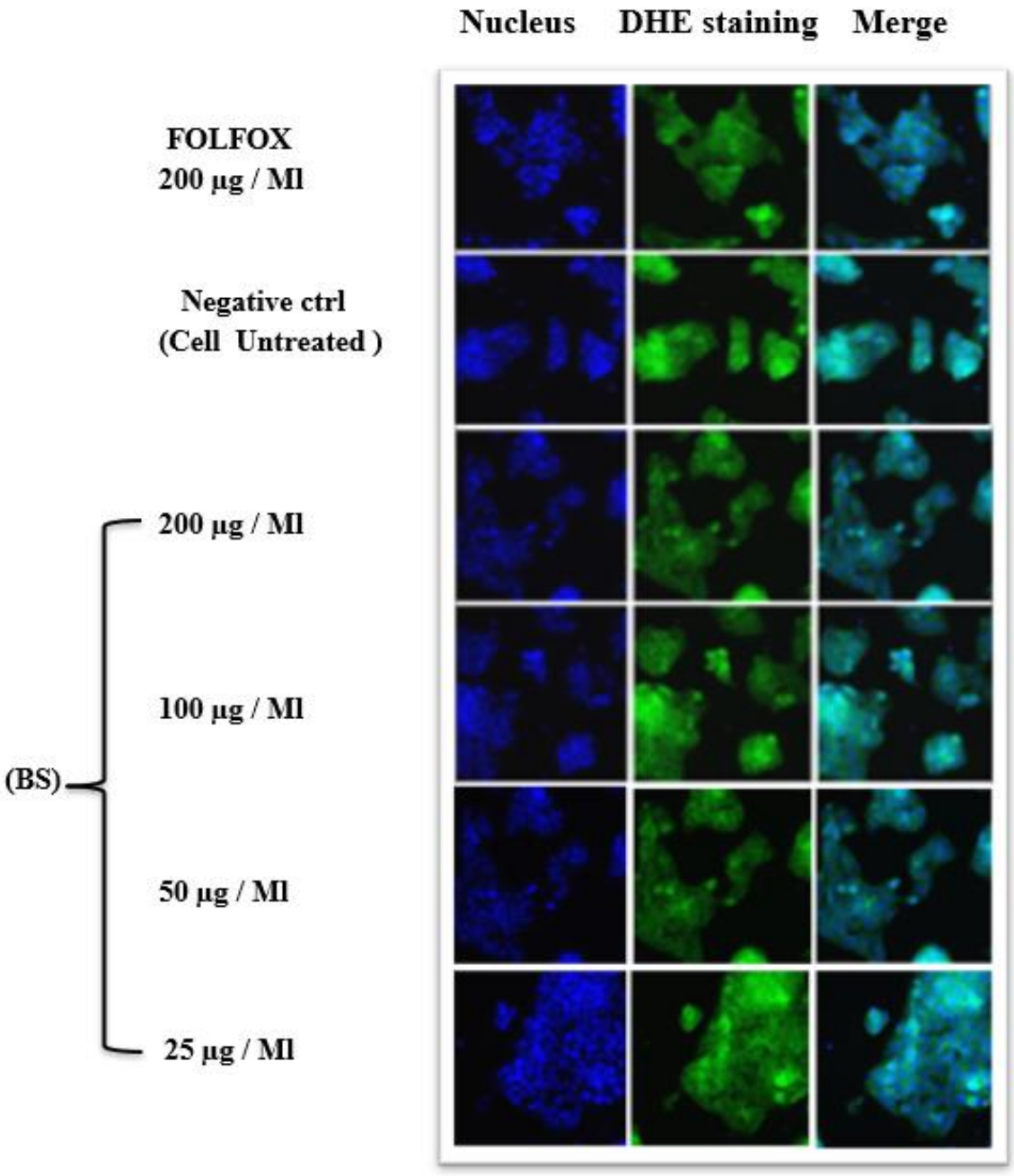
Antioxidants are substances that prevent the oxidation of molecules and cause radicals to turn into inactive derivatives , incorrect or ineffective antioxidant mechanisms lead to oxidative stress, which may occur in the presence of many diseases ,such as diabetes, atherosclerosis, inflammatory bowel disease, damage to the heart, brain or transplanted organs, as well as all types of cancer (Żmijewska-Tomczak *et al*.,2014)

Proper functioning of antioxidant mechanisms is also crucial for optimal organ function, so it was important to carefully examine this probiotic extracted from *B. bifidum* to determine its potential antioxidant properties (Asard *et al*.,2004).

4-7-6- Assay Results Reactive Oxygen Species (ROS)

This Assay was conducted using High Content Screening technology to evaluate reactive oxygen species responsible for a wide range of diseases, including cancer. The results showed an increase in the production of reactive oxygen species after 24 hours of exposure to different concentrations of (BS), with the use of untreated cells as a negative control, and the use of FOLFOX chemotherapy as a positive control, as in picture (4-10), which shows the changes that occur in the cells.

The results showed significant differences and an increase in ROS production at concentrations of (200, 100) µg/ml where the increase reached (1.67, 1.42.)%, The results also showed no significant differences between the effect of (BS) at a concentration of 200µg/ml and FOLFOX at a concentration of 200µg/ml, which increased by (1.72)% compared to the negative control as shown in puctur (4-10) ,Table (4-10)A,B and Figure (4-11).



Picture (4-10) ROS Assay Using HCS Technique for [HRT-18] Cells Treated with Different Concentrations of (BS) Compared to FOLOX Chemotherapy and Untreated Cells After 24 Hours of Incubation at 37C° (Staining: Dihydroethidium (DHE), Hoechst 33342 Stain)

Table (4-10) A Results of the (ROS) Assay for [HRT-18] Cells Treated with Different Concentrations of (BS) Extracted from *B. bifidum* and the FOLFOX After 24 Hours of Incubation at 37C°

Parameters	Mean ± SD Fluorescent Intensity (RFU)%						Sig.	*p* Value	LSD (F Value)
	Control	FOLFOX	BS Concentration in µg / mL						
			200	100	50	25			
ROS	458.0 ±17.6	786.7 ±14.8	763.7 ±10.2	650.7 ±10.5	443.7 ±12.5	454.7±7.5	**	<0.001	49.09

Table (4-10) B Fold of Change

Parameters	Fold of Change					
	Control	FOLFOX	BS Concentration in µg / mL			
			200	100	50	25
VCC	1.0	1.72	1.67	1.42	0.97	0.99

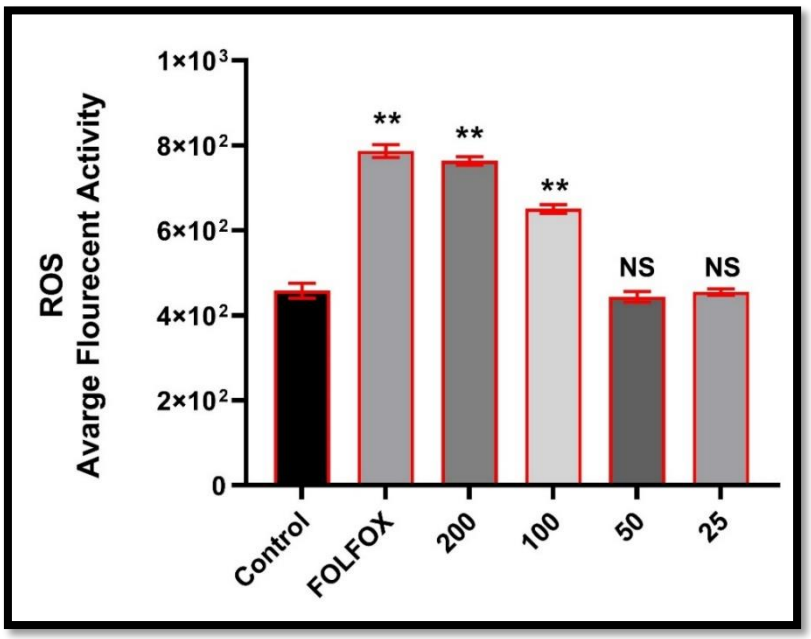


Figure (4-11) Effect of Different Concentrations of (BS) Extracted from *B. bifidum* and FOLFOX on Reactive Oxygen Species (ROS) in the [HRT-18] Cell Line After 24 Hours of Incubation at 37C°

The main sources of reactive oxygen species production in cells are electron transfer reactions catalyzed by mitochondrial systems in adipose tissue, some electrons leak out during this process and react with oxygen molecules, to deal with this natural source of reactive oxygen compounds, tissues have high concentrations of antioxidants such as vitamin C, beta-carotene, and antioxidant enzymes (Hong *et al*., 2024). If too much damage occurs to the mitochondria to be repaired, the cell undergoes apoptosis (Herb & Schramm, 2021).

Much current research is based on the concept that elevated intracellular ROS levels lead to oxidative stress, which in turn activates programmed apoptosis pathways, particularly in cancer cells that are vulnerable to this disruption1 - (Onoue *et al*.,2008).

The results of the current study showed a clear and dose-dependent effect and demonstrated a significant increase in ROS at concentrations of 200 and 100 µg/ml, while no changes were recorded at lower concentrations 50 and 25 µg/ml,

indicating that the effect is dose-related, this result supports the view that higher doses lead to ROS levels that can initiate cell death.

The study noted that (BS) at a concentration of 200 µg/ml showed similar efficacy to FOLFOX treatment, with ROS production increasing by 1.72% in both treatments compared to the control, suggesting that (BS) may work through the same mechanism of cellular oxidative stress that FOLFOX utilises to kill cancer cells.

From this, we conclude that when cells are treated with high concentrations of (BS), ROS is generated in sufficient quantities to disrupt cellular survival pathways through oxidative stress, leading to loss of mitochondrial function, decreased membrane potential (MMP), release of Cytochrome C, activation of Caspase-9/8, and ultimately apoptosis.

A recent study in Frontiers in Endocrinology (2023) confirmed that ‘increased ROS can induce programmed cell death in colon cancer cells by activating internal pathways such as HIF-1α (Nelson *et al*.,2023).

Another study published in Scientific Reports (2021) indicated that biosurfactant promotes ROS generation in a dose-dependent manner, causing a significant decrease in cell viability and integrity even at 200 µg/ml (Panchariya *et al*.,2021).

The results of the current study's ROS Assay were similar to other studies that confirmed that reactive oxygen species (ROS) have a dual role in the cell and can be both destructive and constructive (Nosaka & Nosaka, 2017).

ROS is involved in many activities that regulate the oxidation and reduction process in cells to maintain cellular balance. However, its excessive production leads to oxidative stress, which is a harmful process involved in cell damage and may cause various pathological conditions (Halliwell & Gutteridge, 2015).

Previous research has shown that (ROS) act as potential signaling molecules controlling many normal physiological functions at the cellular level, in addition,

there is a growing body of evidence supporting the role of (ROS) in various disease states. The dual nature of (ROS) with its beneficial and harmful properties suggests its complex role in intervention procedures to treat ROS-associated diseases (Sies,2022).

4-8- Investigation of *NOX4* Gene Expression in Colorectal Cancer Cell Line [HRT-18], Quantitative Reverse Transcription Real-Time PCR (RT- qPCR)

In the current study, Quantitative Reverse Transcription Real-Time PCR (RT-qPCR) assay was performed to measure the quantitative levels of messenger RNA (mRNA) as an indicator of gene expression of the *NOX4* gene in the colorectal cancer cell line [HRT-18], and the *GADPH* gene was used as a standard regulator to calculate gene expressio, by comparing cancer cells treated with different concentrations of (BS) with the negative control (untreated cells). The results showed significant differences ($P \leq 0.0001$) and the occurrence of (Up regulation Ct) and an increase in the gene expression of *NOX4* in the cell line [HRT-18] after treatment with concentrations (400, 200)µg/ml , which amounted to an increase of (26.1, 14.4)% compared to the negative control.

While at concentrations (100 ,5 ,25) µg/ml, no significant differences or increase in gene expression were shown, as given in Table (4-11), Figure (4-12), and Figure (4-13). The Ct value was calculated based on the Livak equation.

Table (4-11) Gene Expression of *NOX4* Gene in the [HRT-18] Cell Line Treated with Different Concentrations of (BS) Extracted from *B. bifidum*, Using Quantitative Reverse Transcription Real-Time PCR (RT- qPCR) Assay

Treatment	Mean ± SD Fold of Change (RFU) BS Concentration in µg / mL						Sig.	*p* Value
	Control	25	50	100	200	400		
	1.0 ± 0.0a	1.32±0.37	1.74±0.42	3.03±0.81	14.42±2.74	26.17 ± 5.51	**	<0.0001
p < 0.05								

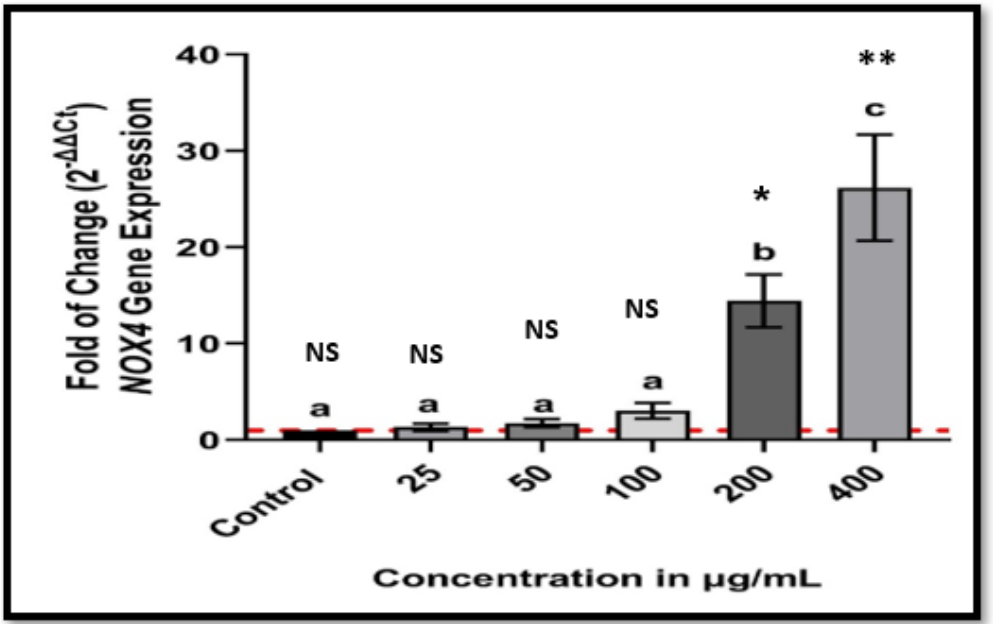


Figure (4-12) Effect of the Different Concentrations of (BS) Extracted from *B. bifidum* on the Gene Expression of *NOX4* Gene in the [HRT-18] Cell Line Using Quantitative Reverse Transcription Real-Time PCR (RT- qPCR) Assay

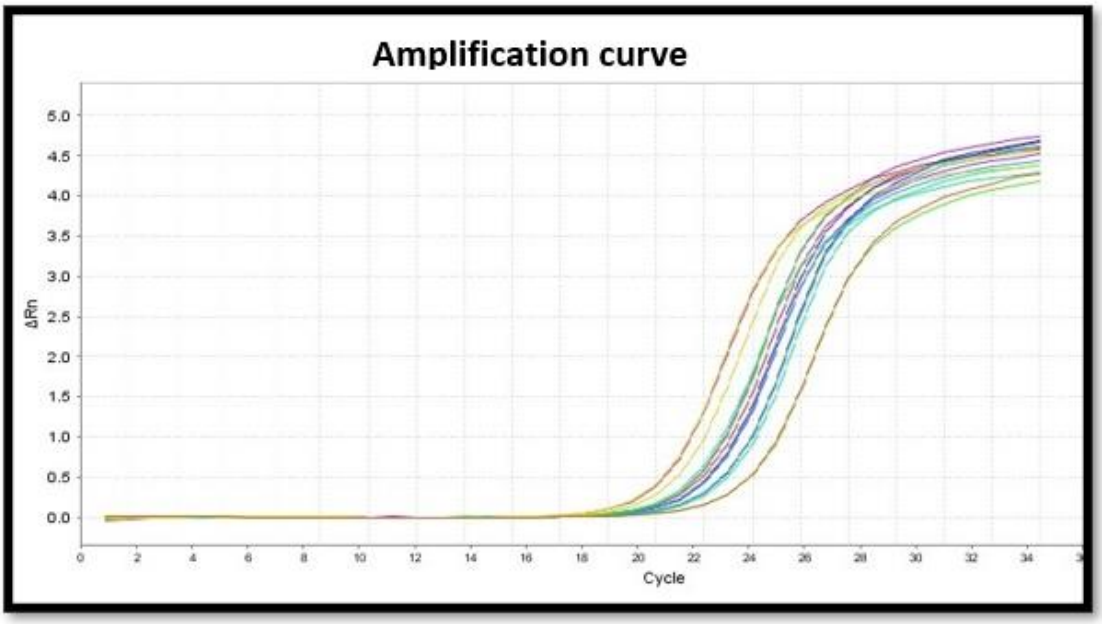


Figure (4-13) PCR Amplification Curve of the *NOX4* Gene in the [HRT-18] Cell Line Treated with Different Concentrations of (BS) Extracted from *B. bifidum* Using Quantitative Reverse Transcription Real-Time PCR (RT-qPCR) Assay

The results of the current study indicate that (BS) at high concentrations 400 and 200µg/ml caused a significant increase in the gene expression of *NOX4* in [HRT-18] cells, while low concentrations did not cause significant changes, we conclude that the effect varies with concentration, this may be due to different mechanisms operating at high concentrations compared to low concentrations. For example, high concentrations may lead to cellular stress that activates *NOX4* as a response mechanism, whereas low concentrations may not be sufficient to stimulate this response.

The current study showed that (BS) has a mechanism of action that causes increased expression of *NOX4*, the effect of (BS) may be direct on the gene transcription of *NOX4*, or it may act through other cellular signalling pathways,

we conclude from these results that (BS) could be a potential therapeutic target (Lin *et al*.,2017).

As for the role of the *GADPH* gene, it is often used as a suitable reference gene for normalising gene expression data, However, some studies have shown that *GADPH* expression can vary under certain conditions, including drug treatment (Munir *et al*.,2024).

A recent study has indicated that certain natural compounds can reduce *NOX4* expression and inhibit cancer cell growth. Comparing the results of the current study could help to better understand the role of *NOX4* in colorectal cancer and how this gene can be targeted therapeutically (Liu *et al*.,2024).

The current study is similar to other studies that used Quantitative Reverse Transcription Real-Time PCR (RT- qPCR) to measure the quantitative levels of mRNA to investigate the amount of gene expression for a specific gene in cancer cells , which confirmed that the onset, development and spread of cancer are due to multiple factors that are linked to each other, and one of these factors is the cellular redox balance supported by the balance between the production and removal of reactive oxygen species (Saikolappan *et al*.,2019).

In addition, redox signals modulate the composition of the extracellular environment, capillary growth, and immune cell responses, and thus contribute to modulating tumor metastatic capacity and therapy resistance (Weinberg *et al*.,2019).

Other research has confirmed that (ROS) has a fundamental role in modifying and maintaining genetic transformation at different levels, as the (ROS) system modifies the renewal process of metabolic pathways of cell lines that are important for the survival and spread of cancer cells (Wang *et al*.,2019).

The NADPH oxidase (NOX-es) family of enzymes is one of the sources of cellular reactive oxygen species(ROS)that play a role in controlling carcinogenic processes (Roy *et al*.,2015), however, disruption of the production of (ROS)

derived from nitrogen oxides, which may be associated with changes in cells due to many disease conditions including cancer (Brandes *et al*.,2014).

The *NOX4* gene is one of the most studied members of the NOX family as it is expressed in most cells, ensuring a role in maintaining the basic physiological redox balance, *NOX4* activity can be enhanced by hypoxia as well as by stimulating mRNA transcription and protein translation (Shi *et al*.,2020).
Other studies have also confirmed the high levels of mRNA in the *NOX4* gene, and its levels have been identified in various cancers (Azouzi *et al*.,2017).

Based on the results obtained, (BS) may be a potential treatment for colorectal cancer. However, the results indicate that (BS) may affect the gene expression of *NOX4*, an important gene in cancer biology.

Chapter Five

Conclusions and Recommendations

5- Conclusions and Recommendations

5-1- Conclusions:

From the results of the current study the following conclusion can be given :

1) *Bifidobacterium bifidum* can be isolated from dairy products, cow's milk, and breast milk, and its identity can be confirmed based on phenotypic tests, biochemical tests, sugar fermentation tests, Vitec identification cards, and molecular diagnosis using PCR.

2) Biosurfactant extracted from *B. bifidum* had cytotoxic effects on the HRT-18 colorectal cancer cell line, causing decreased cell viability and the killing rate increased with increasing concentration.

3) While the Biosurfactant (BS) did not show acute cytotoxicity on normal HDFn cells compared to the chemotherapy FOLFOX.

4) The IC50 of the (BS) against HRT-18 cancer cell line was lower than the IC50 of FOLFOX.

5) When comparing the Biosurfactant (BS) with Ascorbic acid, which is a good standard antioxidant, the results showed no significant difference between (BS) and Ascorbic acid when treated with the HRT-18 colorectal cancer cell line.

6) Biosurfactant (BS) increases the production of reactive oxygen species, which are important in regulating the oxidation-reduction process in cells to maintain cellular balance.

7) *NOX4* gene expression increased in the [HRT-18] cell line with increasing concentrations (BS) and decreased with low concentrations compared to untreated cells.

5-2- Recommendations :

The most important recommendations based on the results of this study are given below:

1) More studies can be conducted on beneficial bacteria to recognise their effectiveness against other types of cancers.

2) Further molecular and genetic studies can be done to identify the effects of *B. bifidum* probiotics on the gene expression of other genes.

3) Extraction and purification of substances produced by *B. bifidum* bacteria and the utilisation of these substances in the fields of food and medicine.

4) Although the results of the current study showed that *B. bifidum* Biosurfactant is effective against cancer cells and has little or no harmful effect towards normal cells, it is preferable to conduct complementary studies to reveal the constituent substances of the Biosurfactant and identify the most effective substances in the process of programmed cell death of cancer cells.

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A

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الخلاصـة :

الدراسة الحالية تضمنت عزل وتشخيص بكتريا *Bifidobacterium bifidum* والتعرف على مدى قدرتها في إنتاج المشتت الحيوي السطحي Biosurfactan (BS)، واستخلاص المشتت الحيوي السطحي منها والتحري عن تأثيره في خط خلايا سرطان القولون والمستقيم [HRT-18] (Human Rectal Tumor)، ثم التحري عن التعبير الجيني لجين NADPH Oxidase 4 gene (*NOX4*) لنفس الخط الخلوي بعد معاملته بالتراكيز المختلفة من المشتت الحيوي السطحي المستخلص ومقارنة تأثير هذه التراكيز مع تأثير العلاج الكيميائي FOLFOX.

إجريت الدراسة الحالية في الفترة الممتدة من بداية شهرنيسان 2024 وحتى نهاية شهركانون الثاني2025 وقد تضمنت ثلاثة محاور أساسية ، المحورالأول عزل وتشخيص البكتريا واستخلاص المشتت الحيوي السطحي منها في مركز الأمين للأبحاث / العتبة العلوية المقدسة ، وفي مركز ابحاث التقنيات الإحيائية / جامعة النهرين ، ومركز مختص بالدراسات الطبية في محافظة بغداد ، المحور الثاني فحوصات السمية الخلوية Cytotoxicity والمحور الثالث التحري عن التعبير الجيني لجين (*NOX4*) تم إجراؤهما في جامعة ملايا كلية الطب/قسم الصيدلة مركز التحري عن العلاجات الجديدة في ماليزيا Center for Natural Product Research and Drug Discovery, Department of pharmacology, faculty of Medicine . University of Malaya , Kuala Lumpur

تم عزل وتشخيص خمسة عزلات من بكتريا *B. bifidum* من مصادر مختلفة والتحري عن قدرة البكتريا في إنتاج المشتت الحيوي السطحي، ثم استخلاص المشتت الحيوي السطحي وتجفيدة بجهاز Lyophilizer، ثم حُضرت خمسة تراكيز من (BS) وهي (25، 50، 100، 200، 400) مايكروغرام/مل

تم الكشف عن التأثيرات السمية الخلوية باستخدام فحص [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (MTT) تم تعريض الخلايا السرطانية بعد تنشيطها للتراكيز الخمسة المحضرة لمدة 24 ساعة وبدرجة حرارة 37م° ، وأظهرت النتائج وجود تأثيرات سمية خلوية للـ (BS) على خط خلايا [HRT-18] إذ لوحظ انخفاض في عدد الخلايا السرطانية مع زيادة في نسبة معدل التثبيط بزيادة التركيزات حيث وصلت اعلى نسبة تثبيط عند التراكيز (400,200) مايكروغرام/مل وقد بلغت النسب (68.4,61.3) %على التوالي في حين لم يظهر(BS) سمية خلوية حادة على الخلايا الطبيعيHuman Dermal Fibroblasts, neonatal (HDFn) وأظهرت النتائج فروقاً معنوية ($P \leq 0.0001$) في حساب الحد المثبط النصفي(IC50) Inhibitory Concentration 50%عند معاملة الخلايا السرطانية [HRT-

18] بالـ(BS) حيث بلغ (53.4) مايكروغرام/مل ، وفي خط الخلايا الطبيعية HDFn قد بلغ (93.2) مايكروغرام/مل .

وقد أكدت النتائج وجود فروقاً معنوية ($P \leq 0.0001$) بين (BS) والعلاج الكيميائي FOLFOX في الخط الخلوي السرطاني [HRT-18] بالتراكيز بين (25 , 400) مايكروغرام/مل ، بينما أظهرت النتائج وجود فروقاً معنوية بين (BS) وFOLFOX في الخط الخلوي الطبيعي HDFn بالتراكيز(400,200) مايكروغرام/مل ولم تظهر أي فروقاً معنوية بالتراكيز (25 ، 50 , 100) مايكروغرام/مل.

وعلى ضوء نتائج MTT تم أجراء فحص High Content Screening (HCS) لتقييم السمية الخلوية وتضمنت النتائج قياس عدة مؤشرات خلوية ، وذلك من خلال الكشف عن التغيرات التي تطرأ على الخط الخلوي السرطاني [HRT-18] بأستخدام التراكيز(25 ، 50 ، 100 ، 200) مايكروغرام/مل من الـ (BS) وتم مقارنة جميع المعاملات مع السيطرة السالبة (الخلايا غير المعاملة Untreated) والسيطرة الموجبة (العلاج الكيميائي FOLFOX) بتركيز(200) مايكروغرام/مل ، حيث أكدت نتائج الفحص (HCS) وجود تأثيرات معنوية على معظم المؤشرات الخلوية بزيادة تركيز الـ (BS) على الخط الخلوي السرطاني [HRT-18] وخاصتاً في التراكيز(200،100) مايكروغرام/مل مقارنة مع السيطرة السالبة و أوضحت النتائج عدم وجود فرق معنوي بين تأثير الـ (BS) و FOLFOX بالتركيز (200) مايكروغرام/مل.

وأظهرت نتائج فحص Caspase-9 and Caspase-8 إن Caspase 9 قد أظهر زيادة معنوية مقارنةً بالسيطرة السالبة وكانت هذه الزيادة معتمدة على التراكيز التي تتراوح بين (25 ، 200) مايكروغرام/مل ، بينما Caspase 8 أظهر نشاط أقل مقارنة مع Caspase 9 ، مما يشير إلى أن المسارالأساسي في عملية الموت المبرمج هو المسار الداخلي والمسؤول عنه Caspase 9 ، وأظهرت النتائج عدم وجود فروق معنوية بين تأثير(BS) وFOLFOX بالتركيز (200) مايكروغرام/مل مقارنةً بالسيطرة السالبة في كلا المسارين

وأظهرت نتائج فحص Anti-Oxidant Activity (DPPH) 2,2-Diphenyl-1-picrylhydrazyl عدم وجود فروق معنوية بين (BS) وAscorbic acid عند معاملتهما مع الخط الخلوي [HRT-18] مع وجود فرق معنوي ضئِيل في تركيز (50) مايكروغرام/مل.

كما وأظهرت نتائج فحص مركبات الأوكسجين التفاعلية(ROS) Reactive Oxygen Species عن وجود فروق معنوية وزيادة في إنتاج ROS في التراكيز (200,100) ميكروغرام/مل، وأظهرت النتائج

عدم وجود فروق معنوية بين تأثير (BS) وFOLFOX بتركيز (200) مايكروغرام/مل مقارنةً بالسيطرة السالبة.

وأظهرت نتائج التحري عن التعبير الجيني لجين (*NOX4*) في الخط الخلوي [HRT-18] بعد معاملته بتراكيز مختلفة من (BS) باستخدام فحص Quantitative Reverse Transcription Real-Time PCR (RT-qPCR) ، إن (BS) له تأثيرات سمية خلوية مع وجود فروقاً معنوية $P\leq 0.0001$ في التعبير الجيني لجين (*NOX4*) في الخط الخلوي [HRT-18] بعد معاملتها بالتراكيز (200 ، 400) ميكروغرام/مل مقارنةً بالسيطرة السالبة ، بينما في التراكيز (25،50،100) ميكروغرام/مل لم تظهر أي فروقاً معنوية في التعبير الجيني ، أي انه بالتراكيز العالية من الـ (BS) يزداد التعبير الجيني لجين(*NOX4*) و كلما انخفض التركيز يقل التعبير الجيني لجين(*NOX4*) المضاد للسرطان.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جـــامعة كربلاء
كلية التربية للعلوم الصرفة
قسم علوم الحياة

تأثيرالمشتت الحيوي السطحي لبكتريا *Bifidobacterium bifidum* على خط الخلايا السرطاني HRT-18 والتحري عن التعبيرالجيني لجين *NOX4*

اطروحة مقدمة الى مجلس كلية التربية للعلوم الصرفة / جامعة كربلاء

وهي جزء من متطلبات نيل درجة الدكتوراه / فلسفة تربية علوم الحياة /علم الحيوان

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