A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF ÇANKIRI KARATEKİN UNIVERSITY

EFFECT EXTRACTION OF ALKALOIDS FROM ALGE *CHLOROCOCCUM HUMICOLA* IN CELL LINE AND GENE EXPRESSION

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

BY

ZINAH TAHA ABDULHAFEDH AL-SHEIKH

ÇANKIRI

2023

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April 2023

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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Zinah Taha Abulhafedh AL-SHEIKH

ABSTRACT

EFFECT EXTRACTION OF ALKALOIDS FROM ALGE *CHLOROCOCCUM HUMICOLA* IN CELL LINE AND GENE EXPRESSION

Zinah Taha Abulhafedh AL-SHEIKH

Master of Science in Biology

Advisor: Assoc. Prof. Dr. Tarkan YORULMAZ

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April 2023

The first assay study effect of extract on lymphocyte division in human blood and this suggest that this extract for *Chlorococcum humicola* alge lead to capture cell division of lymphoid cell in human blood at mitosis stage at different levels. This block was increased with increase the concentrations, the percentage of blocked cells 16.50 to 98.30% for concentrations 50-800 µg\mL. The second assay including effect of extract on growth of tumor cell lines MCSF7, Hela RD and normal cell line was done by using cytotoxic assay. The findings demonstrated that the cytotoxicity of the extract varied depending on cell type and concentration in different cell lines. With higher extract concentrations, tumor cell line inhibition activity increased. The higher inhibition rate in MCSF7 cell line was 99.54% at the concentration 800 µg\mL and lower inhibition rate was 30.07% in concentration 50 µg\mL, while for Hela the highest inhibition rate was 90.31% in concentration 800 µg\mL and lower inhibition rate was 22.19% in concentration 50 g\mL, the highest inhibition rate for RD cell line was 10.11 % in 800 µg\mL and lower inhibition rate was 0.0 % in 50 g\mL and 9.90 in 400 µg\mL. The all results showed that crude alkaloid extract of *Chlorococcum humicola alge* was induced apoptosis by mitochondrial intrinsic pathway after 24 h of exposure.

2023, 71 pages

Keywords: Cell line, Alkaloid, *Chlorococcum humicola*, Apoptosis

ÖZET

HÜCRE ASTARINDA VE GEN EKSPRESYONUNDA *CHLOROCOCCUM HUMICOLA*'DAN ELDE EDİLEN ALKALOLİTLERİN EKSTRAKSİYONUNUN ETKİSİ

Zinah Taha Abulhafedh AL-SHEIKH

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Tez Danışmanı: Doç. Dr. Tarkan YORULMAZ

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Nisan 2023

Ekstraktın insan kanındaki lenfosit bölünmesi üzerindeki ilk tahlil çalışması, *Chlorococcum humicola* alge için bu ekstraktın mitoz aşamasında insan kanındaki lenfoid hücrenin hücre bölünmesini farklı seviyelerde yakalamasına yol açtığını göstermektedir. Bu blok, konsantrasyonların artmasıyla yükselmiştir, bloke hücre yüzdesi 50-800 µg\mL konsantrasyonlar için %16,50 ila %98,30 idi. Ekstraktın tümör hücre hatları MCSF7, Hela RD ve normal hücre hattının büyümesi üzerindeki etkisini içeren ikinci tahlil, sitotoksik test kullanılarak yapılmıştır. Bulgular, ekstraktın sitotoksisitesinin hücre tipine ve farklı hücre dizilerindeki konsantrasyona bağlı olarak değiştiğini göstermiştir. Daha yüksek ekstrakt konsantrasyonları ile tümör hücre çizgisi inhibisyon aktivitesi artmıştır. MCSF7 hücre hattında en yüksek inhibisyon oranı 800 µg\mL konsantrasyonda %99,54, en düşük inhibisyon oranı 50 µg\mL konsantrasyonda %30,07 iken, Hela için en yüksek inhibisyon oranı 800 µg\mL konsantrasyonda %90,31, en düşük inhibisyon oranı 50 g\mL konsantrasyonda %22,19, RD hücre hattında ise en yüksek inhibisyon oranı 800 µg\mL konsantrasyonda %10,11, en düşük inhibisyon oranı 50 g\mL'de %0,0 ve 400 µg\mL'de %9,90 olarak ölçümlenmiştir. Tüm sonuçlar, *Chlorococcum humicola* alge'nin ham alkaloid ekstraktının, 24 saat maruz kaldıktan sonra mitokondriyal intrinsik yol ile apoptozu indüklediğini gösterdi.

2023, 71 sayfa

Anahtar Kelimeler: Hücre astarı, Alkoloidler, *Chlorococcum humicola*, Apoptoz

PREFACE AND ACKNOWLEDGEMENTS

In the name of Allah, the most gracious, the most merciful. Firstly, thanks to Allah for all his blessing.

I would like to express my deep appreciation and sincere gratitude to my advisor Assoc. Prof. Dr. Tarkan YORULMAZ for creating an opportunity to undertake this work support and encouragement during this work and for preparing my thesis.

Special thank goes to the Dean of the College of Science and to all staff of the biology Department in Baghdad University for providing available facilities and valuable information throughout the study period.

Zinah Taha Abulhafedh AL-SHEIKH

Çankırı-2023

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LIST OF SYMBOLS

%	Percent
g	Gram
L	Liter
min	Minute
mL	Milliliter
ºC	Degrees celsius
sec	Second

LIST OF ABBREVIATIONS

A375	Human melanoma cell line
A549	Adenocarcinoma human alveolar basal epithelial cells
AMJ13	Invasive ductal carcinoma
AO\PI	Acridine orange\propidium iodide stain
ATP	Adenosine triphosphate
BAD	Bcl-2 Associated agonist of cell death
BAK	Bcl-2 antagonist killer
BAX	Bcl-2 associated X-protein
Bcl-2	B-cell lymphoma 2
BID	BH3 interacting domain death agonist
CBDs	Cembranoid-type diterpenes
cdk2	Cyclin-dependent kinase 2
cDNA	Complementary deoxyribonucleic acid
C-myc	Cellular-myelocytomatosis
DNA	Deoxyribonucleic acid
E2F	Expression factor
ECM	Extra-cellular matrix
ER	Endoplasmic reticulum
erb B-1	Epidermal growth factor receptor
GC-MS	Gas chromatography–mass spectrometry
H22	Hepatoma 22 cell line
HCT116	Human colorectal carcinoma cell line
HepG2	Liver hepatocellular carcinoma cell line
HIF	Hypoxia-inducing factor
H-ras	Harvey rat sarcoma virus
HRT-18	Human rectal tumor 18 cell line
HSEs	Heat-shock elements
HSF1	Heat shock factor 1
Hsps	Heat shock proteins
HT-29	Human colorectal adenocarcinoma cell line
IC50	Inhibiting concentration 50
LNCaP	Lymph node carcinoma of the prostate
LPS	Lipopolysaccharide
MCF7	Michigan cancer foundation 7
MDA-MB-231	MD Anderson-metastatic breast 231 cell line miRNAs Micro ribonucleic acid
NCI-H292	Non small cell lung cancer cell lineN-myc Neuroblastomas myelocytomatosis
OGs	Oncogenes

P. aeruginosa	*Pseudomonas aeruginosa*
PAR	Photosynthetic active radiation
PC3	Prostate carcinoma 3
PCR	Polymerase chain reaction
RB	Retinoblastoma
RD	Rhabdomyosarcoma
Rf value	Retardation factor value
RNA	Ribonucleic acid
rt-PCR	Real-time polymerase chain reaction
siRNA	Small interfering ribonucleic acid

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1. INTRODUCTION

Microalgae are a kind of photosynthetic microbe that may be found in both saltwater and freshwater environments. Plankton is the common name for these creatures. Microalgae are the foundation of the aquatic food web since they are the major source of oxygen and the lowest trophic level. Microalgae provide several advantages to us that are essential to our survival. Microalgae defy easy categorization because of their distinct characteristics. There are a few different suggestions about how to group them. Size, pigment content, storage profile, ultrastructural diversity, etc. are often used criteria for microalgae classification (Carr and Whitton 1982). However, there is a straightforward method by which we can tell them apart. Cell size is one criterion that may be used to categorize microalgae. Green algae (Chlorophyta), diatoms (Chrysophyta), fire algae (Pyrrophyta), and eugnoloids (Euglenophyta) are only some of the seven types of microalgae that can be distinguished by looking at their cell walls (Barsanti and Gualtieri, 2018). The major photosynthetic pigments of different microalgae give rise to a wide range of cellular colors.

Cancer ranks high on the list of global health concerns and is a key contributor to the rising death toll among both young and old. According to the World Health Organization, the number of people living with cancer reached 21.2 million in 2021, making it the second most prevalent cause of death worldwide after cardiovascular illnesses (Krupa- Kotara and Dakowska 2021). Cancers of the breast, lungs, colon, prostate, skin (excluding melanoma), and stomach account for the vast majority of all cancer diagnoses. Surgery, radiation, and pharmacological treatment (which includes chemotherapy, gene therapy, hormone therapy, and immunotherapy) are all common methods for treating cancer, but they haven't been very successful and come with significant side effects.

Pain, inflammation, and cancer are just some of the many conditions that may be managed with the use of medicinal plants. Natural chemicals extracted from medicinal plants are the current focus of a great deal of research on their potential for suppression.

Of a tumor's development or spread. The huge genus Chlorococcum humicola is found across the tropics and subtropics. Phytochemical analysis of Chlorococcum humicola has shown the existence of flavonoids, and the plant's isolation of secondary metabolites such flavonoids and phenolic acids. Anti-inflammatory, antioxidant, antibacterial, and hepatoprotective effects have all been shown in this genus in previous research. The essential oils have also been recognized for their benefits. Graham and Wilcox 2020 report on the cytotoxic effects of Chlorococcum humicola on A431, HeLa, and MCF-7. Chlorococcum humicola may have impacts on colorectal cancer cell lines, however this has not yet been documented. This study set out to determine whether or not methanol extract, alkaloid, and terpenoid fractions exhibited cytotoxic and apoptogenic activities against the HT-29 human colorectal cancer cell line.

1.1 Aims of the Study

- The aim of this research was to determine the growth phase of Chlorococcum sp microalgae isolated from water eyes from north Iraq and find the best time to harvest extraction alkaloids from alge *Chlorococcum humicola*.

- Study extraction alkaloids in Mitotic Index in lymphocytes of humans.

- Effect of extraction of alkaloids alge on cancer and normal cell line.

- Effect of different concentrations of alkaloids from alge *Chlorococcum humicola* extracts on gen expersstion.

- The effect alkaloids extracts in apoptosis cell line.

2. LITERATURE REVIEW

2.1 Microalgae

Algae, being photosynthetic creatures, have the capacity to convert carbon dioxide into food and energy. Microalgae's wide range of ecological features, cellular size and structure, morphology, photosynthetic pigments, storage and structural polysaccharides, and life histories may be traced back to their unique evolutionary histories. Microalgae consist of a wide variety of microorganisms, including prokaryotic and eukaryotic types. Biologists categorize microalgae by on characteristics including life span, cellular makeup, and morphology (Barsanti and Gualtieri 2018).

According to this system, eukaryotic algae are divided into nine different groups, whereas prokaryotic algae are divided into two (Hoek *et al.* 1995). With a great resistance to a vast range of environmental circumstances including light intensity, temperature, pH, turbidity, and O2 and CO2 concentrations, aquatic microalgae may be found in freshwater springs, salt lakes, open water bodies, and even beneath the ice in polar zones. The furthest depth at which microalgae were found to be able to grow was 268 meters below sea level, at an area where the light intensity was just 0.0005% of that at the surface water. Since red light has a lesser intensity than other wavelengths, it is filtered out at this depth, allowing more of the visible spectrum to penetrate the water (Barsanti and Gualtieri, 2018). Chlorophylls a and b are the primary photosynthetic pigments in these organisms. Chlorophyll c, beta- and gamma-carotene, and a number of xanthophylls serve as accessory pigments (Hoek *et al.* 1995). In chlorophyta, starch is the primary storage carbohydrate. Green algae are divided into many categories based on the complexity of their thallus structures. The thallus may have a variety of sizes and shapes, including a unicellular form, a colonial form, or a filamentous form made up of plates of cells. Their absence of a stem, leaves, and conducting tissue is indicative of their basic anatomy. Their double-membraned chloroplasts, which are responsible for their characteristic green color, are unmasked by supplementary pigments. The pigments needed to absorb light and transmit that energy via a series of enzymatic and photochemical processes are housed in chloroplasts, which are photosynthetic

compartments (Barsanti and Gualtieri 2018).

2.2 Algae (chlorophyta)

Chlorophyta is a kind of green algae that has both chlorophylls A and B and uses starch in its plastids as a means of storing energy. Some Chlorophyta species are unicellular, while others have many cells. Within Chlorophyta, genomic architectures vary widely. However, there are certain universal traits shared by all living things. Chlorophyta may be found in both saltwater and freshwater ecosystems as well as on land. Light, carbon, critical nutrients, water quality, temperature, and tidal exposure are only a few of the conditions necessary for living. Ulva, sometimes known as sea lettuce, is an intertidal plant that thrives in a wide temperature range and may dry out at low tide. In spite of the fact that the vast majority of chlorophyta are found in water:

1- General characteristics

- tend to thrive in the summer, when there is enough of sunlight, nourishment, and heat.

- Some types of filaments could not be edible.

- Flagella may lessen the propensity to drown.

- Planktonic Chlorophyta come in a wide variety of forms, but they always have at least one trait: high possibility for expansion.

2-Cell Structure and Metabolism

Although most Chlorophyta are single-celled, others are multicellular. There are free-living, colonial, and coenocytic species. The nuclei of filamentous sporophytes have singluar lenticular shapes, and their thick cytoplasm surrounds them. The gametes of

chlorophyta are often biflagellated. The chlorophylls a and b found in Chlorophyta are similar to those found in other green plants. In addition, siphonoxanthin and siphonein provide coloration for certain tropical species. Double-membraned chloroplasts are used to store the starches that are produced during photosynthesis. There's cellulose in the cell wall.

2.3 Use of Light by Microalgae

According to the research of Barsanti and Gualtieri (2018), light is a kind of electromagnetic radiation with both particle and wave characteristics. Broadband or whole solar radiation describes the spectrum of sunlight, which consists mostly of wavelengths between 300 nm and 4000 nm. Light with wavelengths in this range contains a variety of potentially dangerous and mutagenic kinds of energy. Total solar radiation is broken down into ultraviolet (UV), visible (PAR or sight), and infrared (IR or heat) components based on wavelength and energy. Light energy is captured by chlorophyll, carotenoids, and phycobiliproteins in blue-green algae (cyanobacteria) due to the absence of the double-membrane organelle chloroplast (Graham and Wilcox, 2020). Most Phyco Bilisome Structures (PBS) are made up of phycobiliproteins. Cyanobacterial PBS are membrane complexes located on the periphery of the cell that effectively collect light energy and transport it to the photosynthetic reaction centers. Thirty percent of the protein in cyanobacterial cells is found in PBS, which is organized into two domains (Grossman *et al.* 2001).

2.4 Chromatic Adaptation

Many types of photosynthetic bacteria and eukaryotic algae are able to adjust their light gathering capabilities in response to changes in the surrounding light regime. There has been extensive research into the connection between the potential and capability of organisms to receive and capture light energy and a wide range of processes, including the initiation of gene expression, the regulation of metabolic and biochemical pathways, and the synthesis and assembly of building blocks for specialized sub-cellular structures (Mouget *et al.* 2004). In particular, cyanobacteria have perfected a light harvesting

mechanism that allows them to make the most of the light's whole spectrum (Cohen & Gurevitz, 2006). Some microalgal species may modify their photosynthetic pigment system by shifting the relative positioning of several components of the apparatus that captures light and converts it into chemical energy. The molar ratio of phycobiliproteins (the pigmented protein inside phycobilisomes) is altered throughout the "complementary chromatic adaptation" process to optimize the cyanobacteria's light uptake capability. Molecular biologists have investigated the role of genetic regulation and full phycobilisome turnover in chromatic adaptation in cyanobacteria. Understanding the genes involved in the adaptation to different environmental circumstances may be gained by studying the production of phycobiliproteins (Chen *et al.* 2022).

2.4.1 Culture parameters

The ideal conditions for microalgae cultivation occur in an artificial setting that closely resembles their natural habitat. There are three main parts to a culture: the nutrient-rich culture media, the algal cells growing in the medium, and the air used to mix the medium with the atmospheric carbon dioxide. Light, temperature, pH, turbulence (mixing), nutrients, and contamination are some of the most critical environmental factors in cultivation. Microalgae species with differing levels of adaptability to environmental change place different weights on various parameters (Chen *et al.* 2022).

2.4.2 Light

Microalgae get the majority of their energy needs from the light they receive (intensity, spectral quality, photoperiod). According to research by Barsanti and Gualtieri (2018), light intensity is a major limiting factor for microalgae development. There have been a number of studies looking at ways to alter some strains of microalgae to increase their tolerance to light intensity and decrease their risk of being inhibited by it (a condition known as "photoinhibition"). Research has shown that decreasing the size of the chlorophyll antenna or the number of light collecting complexes in a chloroplast may both reduce the amount of light absorbed by that chloroplast (Radakovits, 2010). The

proposed method may have two benefits for algal cultures: first, it may allow for more light penetration in high density cultures, and second, it may allow for greater photosynthetic efficiency by reducing the likelihood of photoinhibition in cells that are actively engaged in light harvesting. The effects of different light wavelengths on microalgae development have been extensively investigated, with a focus on the red and blue ends of the spectrum. Not all species have the same photoperiod. Many organisms cannot survive in permanently lit environments, however certain phytoplanktons can (Schenk *et al.* 2008).

2.4.3 Temperature

The best temperature for microalgae cultivation is one that is consistent with the range in which the species occurs in nature. Barsanti and Gualtieri (2018) found that the optimal temperature range for the majority of investigated species was between 16 and 27 degrees Celsius. Temperatures below 16 degrees Celsius may stunt the development of certain species, while temperatures over 35 degrees Celsius will kill them. Changes in the culture temperature may have an effect on the metabolic rate, nutritional needs, and cellular composition of microalgae. Overheating is a significant issue in closed culture systems (photobioreactors) that may be caused by variations in temperature (Richmond 1999).

2.4.4 pH

Although certain species have a great tolerance to very acidic or basic environments, the majority of microalgae thrive in a pH range of 8.2 to 8.7 (Barsanti and Gualtieri, 2018). Since pH has such a profound effect on the biology of the medium, keeping it within the optimal range is crucial. The pH of the medium is crucial to the biochemistry of the cell's metabolism and the uptake of various ions. Because of their ability to counteract the effects of exogenous buffering agents, these two elements have a major impact on algal cultures. Regulated CO2 dissolution has been described as the most cost-effective and practicable strategy for pH control in both heterotrophic and photoautotrophic cultures (Schenk *et al.* 2008). In addition, micro-injection of powerful alkalis and acids

has been used in heterotrophic cells to regulate pH. Another crucial aspect of keeping a culture at peak condition is the mixing models used between the injected gas, liquid (media), and solid particles of algae cells. The homogeneity of the culture can be maintained and nutrients and gas may be circulated through the liquid phase with the aid of mixing (Moheimani 2005).

2.4.5 Nutrient growth media

Carbon, hydrogen, nitrogen, oxygen, phosphorus, magnesium, copper, iron, zinc, sulphur, potassium, molybdenum, and lcium are all important components for microalgae to thrive. The phrase "macro-nutrients" refers to inorganic nutrients eaten by microalgae in large amounts, such as nitrogen and phosphorus (South and Whittick 1987). Growth media should have an abundance of macronutrients. Copper and molybdenum, among others, are needed in trace amounts. Micronutrients are necessary for the proper functioning of the enzymatic system or the electron transport system in algae. Furthermore, vitamins including cyanocobalamin (B12), thiamin, and biotin are crucial for the development of many different kinds of algae. The approximate molecular formula of the microalgae biomass is CO0.48H1.83N0.11P0.01 (Chisti 2007).

2.4.6 Carbon dioxide

Microalgae get the inorganic carbon they need to develop from carbon dioxide. Element analysis of microalgae cells has shown that carbon may account for as much as 50% of cell dry weight (Oh-Hama and Miyachi 1988). As a result, several aeration methods have been researched to learn how different levels of carbon dioxide affect the development of microalgae.

2.4.7 Contamination

The upkeep of a single-species culture is a significant barrier to the large production of microalgal biomass. While open ponds provide a significant danger of contamination, controlled culture techniques make it much easier to keep a single strain of algae. In large-scale microalgal mass production, however, achieving a culture devoid of all contaminants is very unlikely.

As a result, keeping the photobioreactor clean on a regular basis is essential for preventing serious contamination. Yeast, mold, and bacteria are the most common types of biological pollutants found in contaminated cultures (Moheimani, 2005). The pollutants lower both the reliability and productivity of the culture. In open ponds, myxobacteria and protozoa are common pollutants of cyanobacteria cultures (Richmond 1986).

2.5 Classification of *Chlorococcum humicola*

This section has been analyzed under four headings.

2.5.1 *Chlorococcum humicola* alge

Classification of *Chlorococcum humicola* is shown in Table 2.1.

Table 2.1 Classification of *Chlorococcum humicola*

Kingdom	**Plantae**
Phylum	Chlorophyta
Class	Chlorophyceae
Order	Chlamydomonadales
Family	Chlorcoccaceae
Genus	*Chlorococcum*
Species	*Chlorococcum*
Common name	*Chlorococcum humicola*

2.5.2 Description of *Chlorococcum humicola*

Carotenoids and other beneficial substances may be present in chlorococcum, according to some research. It has the potential for outdoor cultivation since it grows successfully in the temperature range of 30 to 35°C and can survive the temperature as high as 45°C. During the hatchery of commercially significant species like shrimp and tilapia, zooplanktons are given chlorococcum for protein and carotenoid (Anjos *et al.* 2013).

As a result, growing Chlorococcum for its high biomass output is crucial. Extraction alge in the of various microalgae, such as Chlorella, Haematococcus, Entomonies, and Chaetoceros, have been shown to produce more biomass when grown in airlift photobioreactors as opposed to stirred tank and bubble column photobioreactors (Moheimani 2005). Liquid mixing and relatively high gas-liquid mass transfer were associated to better performance of airlift systems. Aeration rates from 0.2 to 1.6 vvm were used in our prior research to cultivate Chlorococcum in internal-loop airlift photobioreactors. Despite considerable wall-growth biofilm being plainly evident on the surface of photobioreactors over the whole range of aeration rate indicated, the greatest biomass concentration was achieved at 0.8 vvm. Moreover, when the aeration rate was higher than 1.2 vvm, biofilm development was more widespread, notably on the draft tube and the bottom area of the photobioreactor. Overall biomass production was negatively impacted due to the presence of wall-growth biofilm, which blocked light reaching suspended cells and necessitated repeated aeration stoppages to remove biofilm. Despite the fact that Chlorococcum can grow on the surface like other commercial algal strains (e.g., H. pluvialis, C. vulgaris, and Arthrospira platensis), the

development of a Chlorococcum cultivating system should proceed in suspension rather than an attached-growth system due to better gas and substrate mass transfer and, furthermore, the tendency of Chlorococcum to flocculate so that the conventional biomass harvesting methods like (Converti *et al.* 2009).

Another strategy for removing biofilm was the use of suspended carriers to generate scouring near the surface. While reports for live cell systems like algal photobioreactors are uncommon, this approach is effective, simple to use, and has minimal operating cost. However, its applicability is mostly limited to membrane bioreactors for water and wastewater treatment. Previous works have demonstrated successful algal biofilm removal; however, a number of operational challenges have been reported, including: carriers-liquid separation following cultivation; carriers becoming trapped in bend sections of the photobioreactor; and damage to the photobioreactor's transparent surface (Grima *et al.* 2003). The results demonstrated the significance of selecting suitable suspended carriers for a certain photobioreactor layout. Chlorococcum-specific data on the efficacy of using suspended carriers to clean algal biofilm from photobioreactor surfaces is lacking. Therefore, this research is required to provide justification for this strategy and establish the operational parameters, including the amount of suspended carriers and the aeration rate, essential to keep carriers moving efficiently in photobioreactors. Green algae in fresh water The species Chlorococcum humicola (C. humicola) (Chisti 2007).

2.5.3 Chemical composition of *Chlorococcum humicola*

Physicochemical characteristics of aquatic organisms varied across different bodies of water. The quantity and quality of aquatic vegetation in a given body of water is dependent on a number of factors, including the water's temperature, oxygen level, pH, organic and inorganic matter content, and pollution levels. Green algae are a kind of aquatic plant that plays a crucial role as a photosynthetic producer in the global environment. To present, 101 species of Chlorococcales have been discovered in India, distributed among 18 different taxa (Grima *et al.* 2003). Many species with very dissimilar morphologies were grouped together under the umbrella of Scenedesmus

Meyen. Oxygen from this process is used to aid bacteria in their digestion of organic waste, which in turn aids in the destruction of other dangerous compounds in the sewage purification process. Primary producers in aquatic habitats include chlorococcum, which are tiny, planktonic, greenish, and coccoid algae (Harun *et al.* 2010). Taking into account the Chlorococcales' ecological preferences, it has been shown that lower concentrations of nitrates and phosphates (the two primary nutrients) promote optimal development. The water's temperature is a crucial physical component that modulates the water's other chemical and biological properties. The frequency and distribution of Chlorococcales depend critically on elevated temperatures. Alkaline pH is favorable for algal development, making it a critical ecological characteristic that impacts the survival of aquatic vegetation. Summer and early spring are when you'll see the most Chlorococcales algae. The primary objective of this research was to record and publish the taxonomic and limnological details of the Chlorococcalean green algal flora in Hooghly, West Bengal, India. This research was conducted in West Bengal, India since there is a lack of data on the taxonomy and biodiversity of algae in connection to ecological factors affecting water bodies in the area (Go *et al.* 2012).

2.5.4 Medical importance of alkaloids Chlorococcum humicola

The structural integrity of human cellular macromolecules including DNA, proteins, and lipids is constantly threatened by both endogenous and external factors. A number of toxic compounds produced by humans have found their way into the natural environment. Environmental pollutants such pesticides, metals, PAHs, solvents, and alkylating agents have been demonstrated to cause significant toxicity at specific areas when exposed acutely or chronically. Lack of antioxidant defense in the body may lead to oxidative stress, which is the negative impact. The DNA in a cell is continually under attack from reactive oxygen species (ROS), which are created in the cell as a byproduct of metabolic activities. Multiple oxidant species have been shown to be capable of generating cancer-causing DNA lesions called promutagenic lesions. DNA damage caused by reactive oxygen species (ROS) has been linked to aging and other human illnesses, including atherosclerosis, cancer, neurological disorders, and AIDS.

2.6 Tumors

Tumors are pathological cellular growth disturbances defined by uncontrolled and aberrant cell division. Tumors may be solid masses of tissue or fluid-filled masses of tissue. Benign tumors are those whose cell proliferation is confined to where it began and whose morphology is otherwise unaltered (Sinha 2018).

The expansion of cells is controlled very precisely. To keep it under control, you need to strike a balance between encouraging anabolism and discouraging catabolism. Cellular differentiation and the establishment of cell phenotypic rely heavily on the work of many transcription factors. Multiple signaling pathways control these variables, which in turn control a wide range of physiological and pathological processes and events, including cancer and other disorders (Farhan *et al.* 2017).

2.6.1 Types of tumors

Causes of Benign Tumors The human body regularly generates new cells to replace dying ones. Tumors grow when DNA is broken during cell division and the resulting aberrant cells proliferate faster than the immune system can destroy them. A benign tumor is a collection of cells that does not infiltrate nearby tissues or metastasize (spread to other parts of the body). A benign tumor is not bothersome if it is not putting pressure on neighboring tissues, nerves, or blood vessels and causing harm because of its slower development rate compared to malignant tumors (Wan *et al.* 2016).

Cancerous tumors are referred to as malignant tumors. Mutations in the tumor's DNA or RNA cause proteins to be produced that are either not functional or cause normal cell components to proliferate uncontrollably (Yang *et al.* 2019). Adjacent tissues may be invaded by malignant tumors. Some cancer cells are able to invade the lymphatic system or the circulatory system, where they may rapidly proliferate and metastasize to other organs (Sinha, 2018). These tumors develop abnormally because they may have an aberrant number of chromosomes, have abnormal alterations inside their

chromosomes and on their surfaces, and fail to adhere to their surrounding cells (Vargas-Rondón *et al.* 2017).

2.7 Causes of Cancer Development

For cancer to form, cells must go through a series of changes throughout time (Buder *et al.* 2019). Cancer has many causes, both internal (inherited mutations, hormones, and immune changes), and external (environmental) (smoking, alcohol consumption, physical inactivity, radiation, chemicals, infection with microorganisms, overweight, and poor diet) (Smith *et al.* 2018). These stimuli may set off a cascade of events that culminate in cancer, including aberrant cell proliferation, DNA damage, and a shift in the regulatory circuits that keep cells in check (Rea *et al.* 2018).

2.8 Carcinogenesis

Acquiring the genetic and epigenetic alterations that lead to cancer is a multi-step process (Stewart 2019). mutations in the genes responsible for cell division may cause cells to stop responding to regulatory signals, giving them a competitive edge over neighboring cells (Lopez *et al.* 2021). As shown in Figure 2.1 below, there are three separate phases to this process: induction, dissemination, and advancement (malignant conversion) (Basu 2018).

2.8.1 Initiation stage

This stage results from a spontaneous mutation in the genes, or mutations resulting from exposure to a carcinogenic agent and leads to dysregulation of biochemical pathways associated with proliferation, differentiation, and survival of cells (Basu 2018). The exposed cell to one of the carcinogenic factors, will sufficient from a change in its genetic material, such as the breakdown of one strand of the double helix a, which affects the biological function of the cell and turns it into a mutant cell (Barnes *et al.* 2018).

2.8.2 Promotion stage

It is a selective clonal expansion of initiated cells into a detectable cell mass that is either benign or preneoplastic (Malarkey *et al.* 2018). In this stage, the mutated cells are exposed to external factors called promoters to induce a complete neoplastic by a process called transformation (Stewart 2019). During this time, cancer cells may acquire mechanisms (immunosurveillance escape mechanisms) that enable them to evade detection by immune cells (Kurkowiak *et al.* 2021). The time that the normal cell needed to transform into a mutant cancerous cell and then develop into a noted tumor is called the latency period, and this period varies according to the type of cancer, it may last for a month or even a year (Iglesias *et al.* 2017).

2.8.3 Progression stage

Increases in tumor heterogeneity, invasion, immune evasion, and cell dispersion define the most important stage of cancer development, which occurs when malignant cells invade neighboring areas (Gil Del Alcazar *et al.* 2020). In this stage, the cancer cell becomes more virulent by having a property called metastasis, where the cancer cell separates from the primary tumor and breaks down the proteins that make up the Extra-Cellular Matrix (ECM) that separates neighboring cells from each other, and by destroying these proteins, the cancer cells it can penetrate the ECM and leave the tissue (Strobl *et al.* 2020). These cancer cells then enter the blood and lymphatic vessels and are transported to more distant sites within the body (Follain *et al.* 2020) (Figure 2.1).

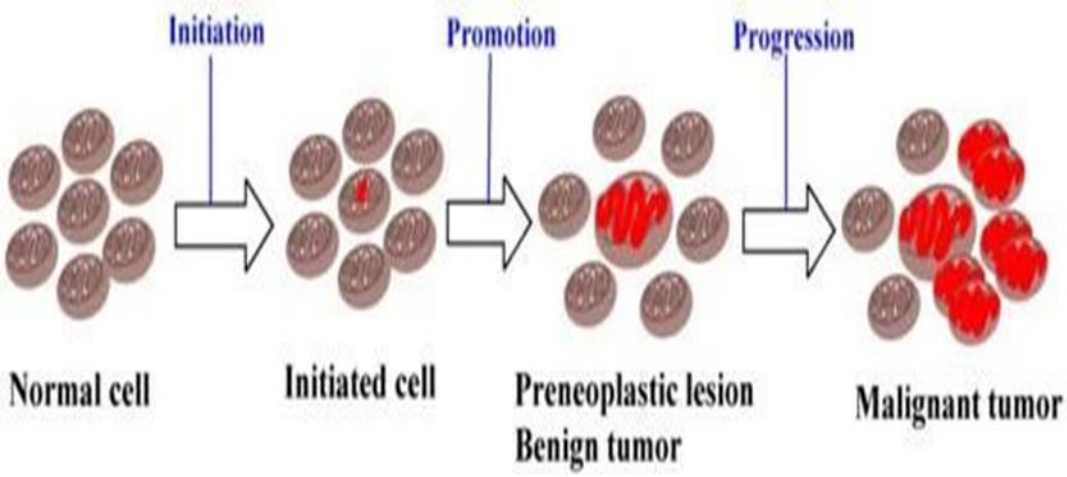

Figure 2.1 Distinct stages of carcinogenesis (Basu 2018)

2.9 Relationship of Cancer with Genes

Cancer develops when a cell's genes get damaged, causing it to proliferate uncontrollably. Tumor suppressor genes, oncogenes, and DNA repair genes are among the many genes crucial to cancer's initiation and development (Romero-Laorden and Castro 2017).

2.10 Gene Expression

Proteins (hormone, enzyme, or receptor) are the end result of gene expression, which is the process by which a gene's instructions are translated into a phenotype. Multiple layers of regulation make this process possible, making it possible for cells to react to changes in their environment (Buccitelli and Selbach 2020). Modulation of gene expression is a fundamental property of all living organisms allowing a cell's protein composition to be adjusted in response to a variety of environmental signals (Pascual-Ahuir *et al.* 2020).

2.11 Programmed Cell Death (Apoptosis)

Cell retention is a highly controlled process that typically takes place throughout growth and aging. When cells are injured by toxic chemicals or illnesses, it may serve as a protective mechanism (Kabel *et al.* 2016). Some hormones (e.g. Corticosteroids) may induce apoptotic death in numerous cells, and many different types of drugs and irradiation can cause DNA damage, which in turn triggers apoptosis. Apoptosis occurs in damaged cells as a result of exposure to carcinogenic factors or when infected with viruses and it is genetic systems that facilitate the destruction of abnormal cells, and then it is one of the mechanisms of inhibition of cancer cells if these cells are exposed to plant extracts, (Kowalczyk *et al.* 2019).

2.12 Heat Shock Proteins and Cancer

Miller and Fort (2018) describe heat shock proteins (Hsps) as a vast family of molecular chaperones that aid in protein folding, degradation, and other processes. These proteins are activated to fix other proteins that have been damaged, protecting the viability of the cell and increasing its resistance to oxidative stress, hyper and hypothermia, and other stressors (Moura *et al.* 2018).

Cancer-related activities that hsps are involved in include cell proliferation, metastasis, and resistance to anti-cancer drugs (Yun *et al.* 2019). Heat-shock proteins are encoded by genes whose heat-shock elements (HSEs) are activated when the heat-shock factor (HSF1) interacts with them (Zatsepina *et al.* 2021).

3. MATERIALS AND METHODS

3.1 Materials

The equipment used is given in three parts.

3.1.1 Equipment and apparatuses

The Equipment and apparatuses were used in this research are shown in the Table 3.1.

Table 3.1 Equipment and apparatuses

Equipment and Apparatuses	Manufacture company	Origin
96-well microtiter plate	SantaCruz	U.S.A
24-well microtiter platE	SantaCruz	U.S.A
Spectrophotometer	Shimadzu	Japan
Refrigerator	Indesit	Turkey
ELISA	Quik Fit	Germany
Soxhlet	Sci-plus	U.K
Electrophoresis	Advance	Japan
Nucleic acid extractor (Anatolia gene works)	Anatolia gene works magnesia 16	Turkey
Micro centrifuge	Hermal	Germany
Cooling centrifuge	Hettich	Germany
Nano drop spectrophotometer	Thermo Scientific	U.S.A
Real-Time polymerase chain reaction	Agilent MX3005P	Germany
Distiller	Kottermann	Germany
Incubator	Memert	Germany
CO2 incubator	Gallenkamp	England
Shaker incubator	Selecta	Spain
Oven	Memaret	Germany
Plastic flask for tissue culture	Falcon	U.S.A
Laminar flow hood	K & K	Korea
Digital microscope camera	Lamin	U.S.A
Rotary evaporator	Memmert	Germany
Micropipette	Volac	England
Deep freezer	The Electron oporation	U.S.A
Inverted microscope	Oltmpus	Japan
Vortex	Hermal	Germany
Florescent microscope	Lumin	U.S.A
Magnetic stirrer	K & K	Korea
PH-Meter	Radiometer	Denmark
Autoclave	Gallenkamp	U.K
Sensitive Balance	Sartorius	Germany
Filter unit 0.22 µm	Microlab Scientific	China
Glassware of different sizes	Meheco	China
Disposable Petri-dishes	Meheco	China

3.1.2 Biological and chemical materials

Table 3.2 lists the chemical and biological substances that were used in this study.

Table 3.2 Biological and chemical materials

Materials	Manufacture company	Origin
Agarose	Sigma	U.S.A
Culture Media (RPMI-1640)	Chemical Point	Germany
Phytohaemagglutinin (PHA)	Medicago	Sweden
Di-Sodium Hydrogen-O-Phosphate (Na2HPO4)	Quali Kems	India
Mono-potassium Phosphate (KH2PO4)	GCC	UK
Potassium chloride (KCl)	Alpha Chemika	India
Fetal bovine serum (FBS)	Avonchem	UK
Giemsa stain	Chemical Point	Germany
Ethanol	Sigma	U.S.A
Methanol	Chem-Lab	Belgium
Chloroform	Chem-Lab	Belgium
Petroleum ether	Universal reagent	India
Colchicine	Avonchem	UK
Acetic Acid	Avonchem	UK
Ammonia (NH3)	Hi-media	India
Sodium bicarbonate	BDH	England
Trypsin\Versine	Sigma	U.S.A
Crystal violate	BDH	England
Trypan blue stain	BDH	England
Phosphate buffer saline (PBS)	Sigma	U.S.A
Formaldehyde	Fluka	Switzerland
Blood agar	Mast Group	UK
Mueller Hinton agar	Hi-media	India
MacConkey agar	Bio-mark	India
Densichek	Bio Meneux	U.S.A

3.1.3 Ready-made diagnostic kits

The ready-made diagnostic kits that were used in this research are shown in the Table 3.3.

Table 3.3 Ready-made diagnostic kits

Diagnostic Kits	Manufacture company	Index No.
Acridine Orange\Propidium iodide staining (AO\PI) kit	US biological (U.S.A)	LGBD10012
ExCellentCT lysis kit	Abm (Canada)	G915
First Chain cDNA synthesis kit	TonkBio (U.S.A)	TB30001B
Kapa express ladder	Kapa (U.S.A)	KK6304
Kapa syber® qPCR master mix kit	Kapa (U.S.A)	KR0389
Magnesia Genomic DNA large volume kit	Anatolia (Turkey)	AE1051

3.2 Selection Algae *C. humicola*

C. humicola are single-celled, round, non-motile organisms with smooth cell walls. Cells may be found either in colonies or alone; their diameters range from 48 m to 58 m; and their chloroplasts are shaped like hollow spheres Figure 3.1.

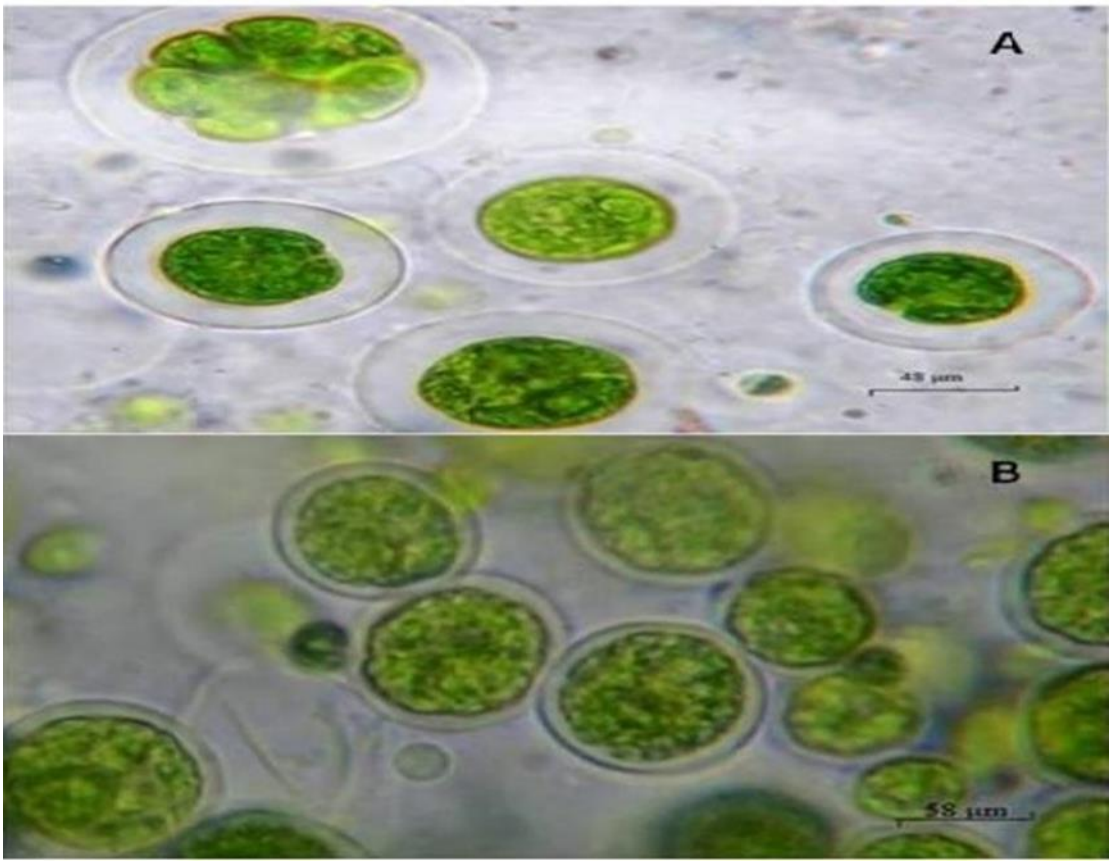

Figure 3.1 *C. humicola* for biomass production of alga A and B in microscope

3.2.1 Media of alge *C. humicola*

All the containers were kept in an incubator with a constant 8000 Lux of light, 24 2 degrees Celsius of temperature, and pH 7.30. The productivity was evaluated after 30 days of development. After the incubation time ended, the biomass was extracted using a centrifuge, and the resulting solid biomass was air dried. Biomass growth rate expressed as a percentage per day and per liter. The samples of water were tested for temperature and acidity. After centrifuging the pure culture, 100 mg of fresh biomass was injected and grown in one liter flasks using Bold's Basal Medium (Table 3.4) (Table 3.5) (Table 3.6) (Table 3.7) (Figure 3.2) (Figure 3.3).

Table 3.4 Isolations of *Chlorococcum spp*. from solid medium need (A) a solution compound amount of a certain kind of chemical

NaNO3	20 gr/L
NaH2PO4	40 gr/L
FeCl3.6H2O	2.6 gr
H3BO3	67.2 gr/L
MnCl2.4H2	0,77 gr
EDTA titriplek III	90 gr
Aquadest	1000 mL

Table 3.5 (B) the number of compounds in solution ideal for isolating *Chlorococcum* spp.

ZnCl2	2.1 gr
CoCl2.5H2O	2 gr
(NH4)6.Mo7O24.4H2	0.9 gr
CuSO4.5H2O	2 gr
Aquadest	100 gr

Table 3.6 The nutritional content of the A solution used in Walne's culture media

NaH2PO4.2H2O	20 gr
NaNO3	100 gr
Na2EDTA	5 gr
Na2SiO3	40 gr
MnCl2.H2O	13gr
FeCl3	10 gr
H3BO3	0.36 gr
Aquadest	1000 mL

Table 3.7 Solution (B) in Walne culture media (trace meta) amount

ZnCl2	21 gr
CoCl2.6H2O	2 gr
(NH4)8.Mo7O24.4H2O	0.9 gr
CuSO4.7H2O	20 gr
FeCl3.6H2O	3.15 gr
Aquades	100 mL

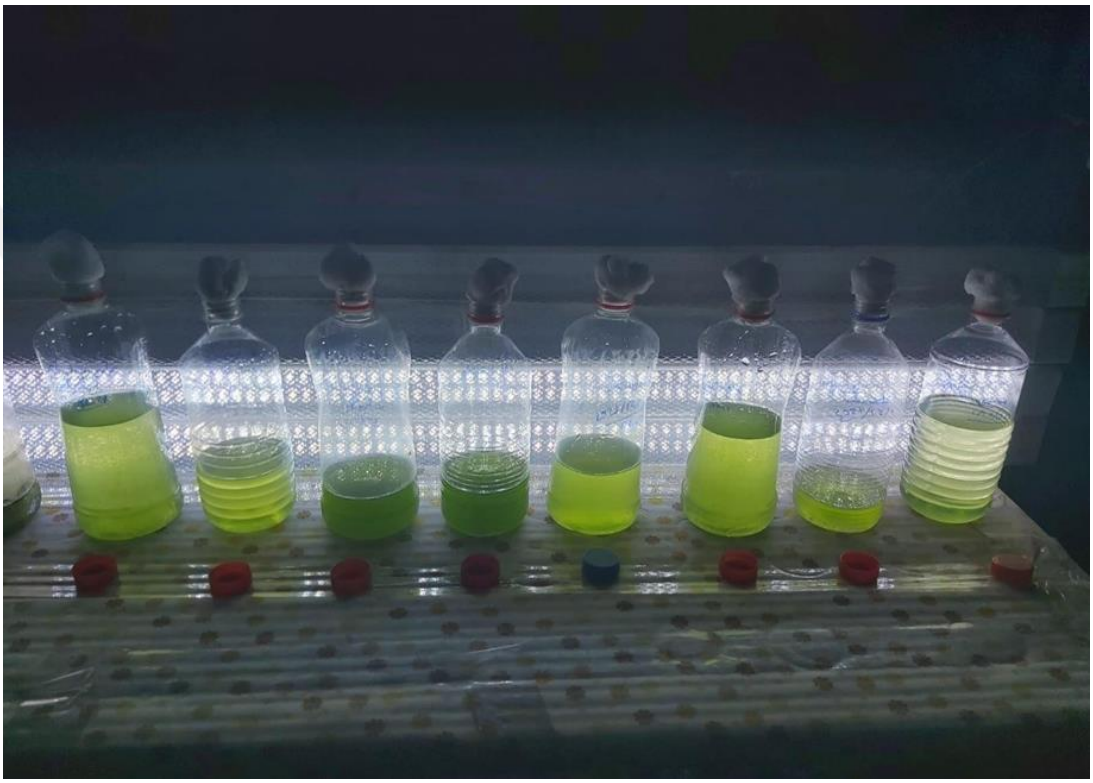

Figure 3.2 *C. humicola* for biomass production of alga in BBM in flasks

Figure 3.3 Culture of *C. humicola* for biomass production of alga in flask

Sodium alginate was used to create synthetic algae. For one hour with constant stirring at 60°C in a water bath, sodium alginate (at a concentration of 4%) was produced in sterile BBM medium. The cells from a one-gram unicultured algae sample were washed in sterile water and then added to a two-volume slurry of sodium alginate. Calcium alginate beads were made by dropping algal cells into a solution of 0.2 M calcium chloride using a pipette. After 30 minutes, the beads had hardened completely. Three to four times, they were rinsed with distilled water. The algal spores were kept cold and dark at 4 degrees centigrade. The beads' vitality was checked at three-month intervals using BBM culture (5 beads per 100 mL culture media). Bagdad University's Biology Laboratory was home to this investigation. The microalgae employed in this study were a strain of Chlorococcum humocola sp. Walne's culture medium had an initial cell density of 187.500 cells/mL. All procedures were performed in a clean room. Both before and after cultivation, microalgae were identified. Ten separate rounds of observation and labeling were performed on each sample. Walne's solutions were employed as a part of the conventional technique for cultivating microalgae. For the purpose of separating Chlorococcum spp. from other species, the composition of particular solid medium is presented. Walne's solutions were added to the liquid medium (to cultivate) after the isolates were transferred.

3.3 Alkaloid Extract fromAlge *Chlorococcum humicola* Preparation

The extract is prepared according to (Harborne 1989) as follows:

- Take a quantity of 10 g of dry plant ground and put it in a cylindrical container made of paper with pores called (Thimble) and put it in the place designated for it in the Soxhlet.

- Add 500 mL of methanol 70% to it and the extraction was carried out for 5 hours

- Pour the methanolic extract into Petri dishes and then the extract was evaporated by using a rotor evaporator at room temperature and then filtrate.

- 100 mL of chloroform was added for every 100 mL of the extract in the separating funnel, to adjust the pH a little weak base (ammonia) was added to get pH: 8 and left for 24 hours, two layers were obtained, the upper layer is the chloroform layer, which was disposed of, and the lower layer is the aqueous layer containing the alkaloid, which was mixed with petroleum ether in equal quantities and then filtered using a 0.22 mm filter unit.

3.4 Detection of Alkaloid

Alkaloid was detected by using Dragendroff's reagent. Further investigation to detect the level of an alkaloid of the plant was performed by an advanced method that can separate each compound in the sample.

3.4.1 Dragendroff's reagent preparation

The reagent is prepared according to (Raal *et al.* 2020) as follows:

Solution A: In a volumetric flask with a 50 mL capacity, we put 0.800 g of BiONO3 and 10 mL of ice-cold HAc. Then, the volume was adjusted by adding distilled water to the mixture.

Solution B: Twenty grams of KI powder were measured out and diluted with water in a 50 milliliter brown volumetric flask. In a 100 mL brown volumetric flask, 5 mL of solution A and 5 mL of solution B were each added before being diluted to volume with distilled water.

3.5 Gene Expression Primers

The primers were used to investigate gene expression during this study, where four genes closely related to induction programmed cell death were selected (BAX, p53) in MCF7, Hela, and RD cancer lines. And three genes related to the phenomenon of resistance of cancer cells to cellular stress conditions were selected, which are the genes encoding the heat shock proteins Hsp90, and HIF in the MCF7 and Hela cancer cell lines. In addition to the Glyceraldehyde-3-phosphate housekeeping (GAPDH) initiator. The primers were designed through this study based on the information available on the Gene bank of Biotechnology Information (NCBI). The sequence of nitrogenous bases for the mRNA deposited at the NCBI website was used for each of the studied genes, and the sequence of the primers was determined using the ApE-plasmid program. Table 3.8 shows the sequence of nitrogen bases in all the primers that were designed.

Table 3.8 Primers used in the study of gene expression

Human Primers	Forward\Reverse	5´-3´ Sequence
P53	Forward	CCG TCC CAA GCA ATG GAT G
	Reverse	GAA GAT GAC AGG GGC CAG
BAX	Forward	CCT CTC CCC ATC TTC AGA TCA
	Reverse	TCA AGT CAA GGT CAC AGT GAG
HIF	Forward	CTC TTG AGC AGT GGC TGG TC
	Reverse	GCT GAT CTA TGA GCG ATA CT
HSP70	Forward	CAA GTG GAC CAG GAG GAA CC
	Reverse	TTC TCG ATT GGC AGG TCC AC

3.6 Concentrations Used for Cytotoxicity Assay

A standard concentration of 100 mg/mL was prepared. Then the concentrations used for the cytotoxic assay were prepared from the standard concentration. Serum-free RPMI-1640 medium was used to make relative dilutions, and five concentrations of 50, 100, 200, 400, and 800 µg/mL were prepared for use in toxicity tests.

3.6.1 Solution used for cytotoxicity testing on lymphocytes

The solutions were prepared according to (Yaseen 1999) in the following Table 3.9:

Table 3.9 Solution used for cytotoxicity testing on lymphocytes

Solution	Preparation Method
Complete Growth Media	RPMI-1640 medium was used with the addition of calf fetal serum. The pH was adjusted to 7.2, then the medium was sterilized using 0.22 µm membrane filters. The medium was incubated at 37°C for three days to ensure that it was free of contaminants, then poured into sterile tubes of 5 mL per tube, and kept at -20°C.
Fetal Calf Serum	was prepared by the Iraqi Biotechnology Company
Phytohaemagglutinin (PHA)	The PHA was prepared simultaneously by dissolving 2.5 mg of PHA in 4 mL of sterile water, taking 0.1 mL of it and adding it to 0.6 mL of sterile water, then taking 0.1 mL of it to be added to each culture tube containing 5 mL of culture medium so that the final concentration reached 15 µg/mL and store at -20°C.
Colchicine	Dissolve 0.5 mg of colchicine powder in 10 mL of distilled water, taking care to prepare it immediately upon use and keep it away from light.
Hypotonic Solution	A hypotonic solution was prepared by dissolving 1.1175 g of KCl in 200 mL of distilled water until the solution had a concentration of 0.075 M, and kept at 4°C until use.
Fixative Solution	Prepared by simultaneous mixing of absolute methanol with glacial acetic acid in a ratio of 1:3 v/v and used cold.
Sorensen's Buffer Solution	Sorensen buffer solution is prepared by dissolving 7.08 g of Na2HPO4 with 6.7 g of KH2PO4 salt in 1000 mL of distilled water and keeping at 4°C until use.
Giemsa Stain	Dissolve 2 g of Giemsa stain powder in 100 mL of absolute methyl alcohol using a magnetic stirrer for 72 hours, then filter the dye using Whattman No.1 filter paper. Keep in an airtight, opaque vial. This solution is stored in stock, and when used, dilute immediately by mixing 1 mL of the dye with 4 mL of Sorensen buffer.

3.6.2 Blood sample collection

Apparent healthy individuals between the ages of 20 and 45 had 5 mL of venous blood extracted from them using a syringe pre-washed with heparin to avoid clotting.

3.6.3 Slides preparation

After being immersed in chromic acid for 72 hours, the glass slides were rinsed with hot water, followed by cold water, and stored in the fridge until use.

3.7 The Effect of Alkaloid in Mitotic Index in Lymphocytes of Humans

The effect of the alkaloid extract at different concentrations on the mitotic index of lymphocytes was studied by using a short-term blood culture, based on the method of (Verma and Babu 1995).

3.7.1 Culture of blood with alkaloid extract

The alkaloid extract was added at concentrations of 50, 100, 200, 400, and 800 µg/mL to the prepared culture tubes containing the complete RPMI-1640 culture medium, and the final volume of the mixture should be 5 mL, and three replicates for each concentration. Then 0.5 mL of blood was added to each tube using a 5 mL syringe, then 0.1 mL of the lymphocyte-cleaving agent (PHA) was added and mixed with the medium gently, and then incubated at 37°C on a tilted form for 24 hours, and the tubes should be mixed every 12 hours. Then a set of tubes was left without any extract added and this treatment was considered a control.

3.7.2 Cells harvest

Each control tube had 0.1 mL of colchicine injected at a concentration of 10 g/mL during the last 150 minutes of the initial culture period. The untreated tubes were

reintroduced to the incubator while the colchicine was not added. After centrifuging each tube for 10 minutes at 1500 rpm, discarding the filtrate, and mixing the remaining culture medium well with the precipitate, we were left with our final product. Each tube was given a warm addition of 0.075 M hypotonic solution at 37°C, ranging from 5-10 mL. The test tubes spent 30 minutes in a 37 C water bath incubation. After 10 minutes of centrifugation at 1500 rpm, the filtrate was thrown away and the tubes were discarded.

3.7.3 Fixation

With vigorous shaking, 5 mL of the precipitate was diluted with a few drops of cold Fixative applied directly to the tube wall. The samples were blended using a Vortex mixer, and then the tubes were stored at 4 degrees Celsius for 30 minutes.

3.7.4 Washing

After centrifuging the tubes for 10 minutes at 1500 rpm, we removed the filtrate and were left with the precipitated cells. The suspension was adjusted many times until a transparent hue developed. 1 mL of the fixative was used to suspend the precipitate, which was then stored at -20°C.

3.7.5 Dropping

The cells were well combined, and then they were dropped on the cold slides at a distance of 0.5-1 m using a Pasteur pipette. The slides were kept clean, cool, moist, and fat-free.

3.7.6 Staining and microscope examination

Slides were stained with prepared Giemsa stain diluted with warm Sorensen buffer solution prepared at a ratio of 4:1 for 2-3 minutes, then washed with Sorensen buffer,

allowed to dry, and then examined by light microscopy to calculate the Mitotic Index (MI) according to (King *et al.* 1982) method. As the cells in the metaphase were divided by the total number of cells examined (1000), as in the following Equation (3.1):

Mitotic Index (MI)={The number of dividing cells\The total number of cells is 1000}*100 (3.1)

3.8 Solutions Used for Tissue Culture

The solutions were prepared according to the method (Giuliano *et al.* 2007) as shown in the following Table 3.10.

Table 3.10 Preparation solutions used for tissue culture

Solution	Preparation Method
Sodium Bicarbonate	The solution was prepared by dissolving 4.4 g of sodium bicarbonate monohydrogen in 100 mL of distilled water, then sterilizing the solution with an oxidizer at a temperature of 121°C for 15 minutes, then keeping at 4°C until use.
Culture Media RPMI-1640	The medium was prepared by mixing 10.4 g of RPMI-1640 with 0.5 mg/L of Penicillin and Streptomycin with 100 mL of fetal calf serum. The components of the medium were mixed and 4.4% of the sodium bicarbonate solution was added and completed the volume to 900 mL by distilled water. Then the pH was adjusted to7.2, then complete the volume to 1 liter with distilled water. The medium was sterilized with a 0.22 µm filter unit and incubated at 37°C for 72 hours to ensure that it was free of contaminants.
Phosphate Buffer Saline Solution (PBS)	Prepare by dissolving 0.85 g of sodium chloride salt in 100 mL of distilled water, sterilization with reflux, and keeping in the refrigerator until use.
Trypsin–Versin solution	This solution was prepared by dissolving 1 gm of trypsin/versin powder in 100 mL of distilled water, adding sodium hydroxide to it to adjust the pH to 7, then sterilizing it with a 0.22 µm filter unit and refrigerating until use.
Crystal Violet Stain	Dissolve 5 g of stain in 200 mL of absolute methanol and filter with Whattman No. 1, then add to the filtrate 50 mL of 37% formaldehyde AND complete the volume with distilled water to 1 liter.
Trypan blue stain	Dissolve 1 gm. of stain in 100 mL of PBS, then filter using Whattman Paper No. 1 and store at 4°C until use.

3.9 Cancer Cell Lines

Michigan Cancer Foundation7 (MCF7): received this line at pass No. 50, and the origin and description of this line were mentioned by the researcher (Soule *et al.* 1973), and

the sample was taken from the breast cancer of a 69-year-old woman. This line was grown in RPMI-1640 medium equipped with 10% calf fetal serum. The cells were treated with trypsin/viresine solution when the complete subculture layer was formed.

Human Cervical Cancer Cell Line (Henrietta Lacks) (Hela): received this line at pass No. 58, and the origin and description of this line were mentioned by the researcher Dr. George Gey in 1951, and the sample was taken from cervical cancer cells of a 31-years-old of African American woman. This line was grown in RPMI-1640 medium equipped with 10% calf fetal serum. The cells were treated with trypsin/viresine solution when the complete subculture layer was formed.

Human Muscle Tissue Normal Cell (Rhabdomyosarcoma) (RD): received this line at pass No. 43, and the origin and description of this line were mentioned by the researcher, and the sample was taken from a pelvic RMS of a 7-years-old female. This line was grown in RPMI-1640 medium equipped with 10% calf fetal serum. The cells were treated with trypsin/viresine solution when the complete subculture layer was formed.

3.10 MCF7, Hela Cancer Cell, and RD Normal Cell Line

The method of (Freshney *et al.* 1997) was used to grow cancerous line cells as follows: Cells of each of the lines were placed in a culture container with a diameter of 25 cm^2 containing RBMI-1640 culture medium and 10% calf B serum.

For 24 hours, a 5% CO2 incubator kept the containers of cell suspension and growth media at 37 degrees Celsius.

- Secondary cultures were performed on the cell culture after a day of incubation, after it was determined that growth had occurred and that it was clean.

- The cells were checked under an inverted microscope to confirm they were healthy, sterile, and contained between 500 and 800 thousand cells/mL.

- The cells were moved to the expansion chamber, and the spent culture media was discarded.

- The cells were washed twice for 10 minutes each with PBS solution before being discarded.

- The cells were treated with trypsin/veresin enzyme for 30-60 seconds at 37°C.

- After collecting the cells in centrifuge tubes, we spun them for 10 minutes at room temperature (2000 rpm/min) to remove the trypsin and the spent growth media, precipitating the cells.

- The cells were resuspended in a new culture medium containing 10% serum, and the filtrate was discarded.

- Count the cells and their viability using a Hemacytometer slide and the Equation (3.2): Take a measured volume of the cell suspension and mix it with the equal amount of Trypan Blue dye:

$C=N\times10^4 \times F\backslash mL \qquad (3.2)$

Since;

- C=number of cells in one mL of solution

- N=number of cells in the slide

- F=dilution factor

- 104=Slide dimensions

The percentage of cell vitality in the sample was also calculated by using a Hemacytometer slide also according to the Equation (3.3):

Live cell viability=(live cells)\(dead cells)x100 (3.3)

The cell suspension was distributed in new containers and then incubated in a 5% CO2 incubator at 37°C for 24 hours.

3.11 Cytotoxic Assay of Alkaloid from *Chlorococcum humicola* on Cancer Cell Lines

Nicotine alkaloid extract was utilized at 50, 100, 200, 400, and 800 μg/mL in five separate experiments. Immediately after preparation, all concentrations were utilized.

- Treat the cells in a 25 cm2 tissue culture container with a trypsin/versine solution, gently shake the bottle to remove any remaining old culture media, and incubate at 37°C for 10 minutes to prepare the cell suspension.

- After 24 hours in the 37°C incubator, the old culture media was removed from the holes and 0.2 mL of the produced concentrations of the extract was applied, with three duplicates for each concentration; • The plate was incubated at 37°C until the cells adhered to the hole;

- Remove the plate from the incubator after 24 hours and add 100 L of crystal violet stain solution to each well containing cells.As only living cells take up the stain, the results were read using ELISA at a wavelength of 492 nm; the plate was then

returned to the incubator for 20 minutes after its contents were removed and the cells were washed with water until the excess stain was removed.

The inhibiting ratio was calculated according to the Equation (3.4):

Percentage of cell inhibition=(absorbance reading of control cells-absorbance reading of treated cells for each concentration/absorbance reading of control cells)x100 (3.4).

Determination of IC50 for each cancer line after an exposure period of 24 hours (Freshney 1997).

3.12 Real-Time Polymerase Chain Reaction

Gene expression measurements were carried out according to the following stages;

3.12.1 Cell seeding stage

Each cancer cell line (MCF7, Hela, and RD) was seeded at a density of one million cells per container in two culture bottles of 25 cm2. For 24 hours at 37 degrees Celsius, the cells were allowed to adhere, proliferate, and form a monolayer in each of the containers.

3.12.2 Exposure stage

One culture bottle of each cell line was left untreated and returned as a positive control after monolayer formation and the cells were subjected to nicotine alkaloid extract for 24 hours at the IC50 concentration for each cell line.

3.12.3 Harvesting cells stage

Harvested cells were collected in sterile test tubes without removing the culture medium, centrifuged to separate the cells, and then stored at -80°C in 50 µL of cooled PBS solution. The culture medium was discarded after the centrifugation step.

3.12.4 RNA Extraction from Cancer Cell Lines (MCF7, Hela, and RD)

Total RNA was extracted from untreated cells (control) and treated with nicotine alkaloid extract using Excellent CT Lysis kit from (Abm, Canada) according to the method of (Mahmood *et al.* 2017). This kite contains a solution to analyze cells, remove DNA, and proteins. The resulting RNA can be used directly for the manufacture of complementary DNA and in Polymerase chain reaction experiments.

The components of the diagnostic kit used for RNA extraction are shown in Table 3.11.

Table 3.11 Components of the diagnostic kit for RNA extraction

Components	Volume
Decomposition solution	1.25*2 mL
Stop solution	300 µL
Protease enzyme	50 µL
Protease inhibitor	50 µL

Procedure

- 1 µL of Protease enzyme was added to every 10-100000 cells to get rid of proteins and mixed with 50 µL of the decomposition solution which is acts on the cell wall and DNA and incubated for 10 minutes at 37°C.

- After the incubation period was over, 1 µL of protease inhibitor solution was added to the mixture to stop the action of the protease enzyme.

- The decomposition was then completed by adding 5 μL of stop solution and the mixture was incubated at room temperature for 2 minutes and transferred to a deep freezer.

3.13 Reverse Transcription (Convert RNA to Complementary DNA (cDNA)

Reverse transcription (cDNA synthesis) was performed by using PCR technology by using the First Chain cDNA Synthesis Kit from TonkBio.

Components of the diagnostic kit for reverse transcription are shown in Table 3.12.

Table 3.12 Components of the diagnostic kit for reverse transcription

Components	Volume
TonkBio™ M-MLV Solution	120 μL
OligodT Primer	120 μL
Reaction Buffer Solution * 5	500 μL
dNTP Mixture (10 μM)	240 μL
RNase Inhibitor Solution	60 μL

Principle

Synthesizing cDNA from the RNA template is simplified using this kit. Reduced RNase H activity in the M-MLV reverse transcriptase used in this kit prevents the breakdown of RNA-DNA hybrids during the cDNA synthesis process.

Procedure

Mix the ingredients in a centrifuge and put them on ice.

- The following ingredients are added in a sterile Nuclease-free tube on ice (Table 3.13):

- Incubated at 65°C for 5 minutes, then cooled on ice.

- Then, it was incubated at 42°C for an hour.

- Finish the reaction by heating to a temperature of 70°C for 5 minutes.

Table 3.13 Ingredient of this step

Ingredients	Volume
RNA/mRNA	1 µL
OligodT primer	1 µL
RNase-Free water	10.5 mL

3.14 Measuring the Concentration and Purity of cDNA

The Nano drop spectrophotometer was used to determine the concentration and quality of the cDNA, with the latter being determined by measuring the optical density ratio (OD) at a wavelength of 260/280 nm, which corresponds to the absorption wavelengths of DNA and protein, respectively. The minimum required concentration of cDNA in a sample is 1.8 (Sambrook and Russel 2001).

3.15 Primers Preparation

Different concentrations of integrated primers were achieved as a dried product. The components were dissolved in sterile, nuclease-free distilled water to yield a stock solution with a final concentration of 100 picomoles; then, 10 L of this solution was added to 90 µL of distilled water to get 10 picomoles of the final concentration of the working solution that was used in the real-time polymerase chain reaction (RT-PCR) method.

3.16 Measuring the Gene Expression of Programmed Cell Death Genes and Genes Encoding Heat Shock Protein

The PCR reaction was carried out by using a Kapa syber green master mix kit from (KAPA, USA). The reaction was carried out in a volume of 20 µL by adding the reaction components shown in Table 3.14.

Table 3.14 Components of real-time polymerase chain reaction

Components	Con.	Volume
KAPA SYBR® FAST qPCR Master Mix Mixture (2X)	1x	10 µL
Forward Primer	10	1 µL
Reverse Primer	10	1 µL
cDNA Template	-	6 µL
Nuclease-Free Water to 20 µL	-	2 µL

By using the thermal cycle program described in the following Table 3.15.

Table 3.15 Thermal cycle program of RT-PCR

Step	Temperature	Period	Cycle
Initial denaturation (DNA polymerase activation)	95 ℃	3 second	Hold
Denaturation	95 ℃	1-3 sec.	40
Annealing	62 ℃	20 sec.	40
Elongation	72 ℃	30 sec.	-
Extention	72 ℃	10 sec.	-

3.16.1 Calculate of gene expression in RT-PCR reaction

The gene (GAPDH) was used as a positive control to calculate the value of the change in the threshold cycle ΔCT. The GAPDH gene is one of the most common genes used in gene expression data collection. The reason for using the GAPDH gene as a positive control in molecular studies is that its expression remains constant in the cells or tissues to be detected (Rebouças *et al.* 2013). The percentage of gene expression for the studied genes in the DNA genetic material was calculated according to the method.

3.17 Electrophoresis of Genetic Material (DNA)

Cells seeding and harvesting of the three cancer cell lines were carried out according to paragraph of 3.12.

3.18 DNA Extraction

DNA extraction was carried out by using Magnesia Genomic DNA Large Volume Kit from (Anatolia, Turkey) according to (Alrawi 2017) method. This diagnostic kit contains all the reaction components needed to purify and extract DNA using the Magnetic-particle technique.

Component of DNA Extraction kit is shown in Table 3.16.

Table 3.16 Component of DNA extraction kit

Component	No. of pieces
Cartridge Prefilled with Solutions	96 Pieces
Automatic Pipette set in Addition to Holder Set	100 groups
Test Tubes	100 Pieces
rinsingTubes	100 Pieces
Proteinase K Enzyme (11mg)	8 pieces
PK Storage Buffer	8 pieces

Cartridge components

The cartridge provides a strong spinning force for the bonding/washing steps and thus gives high purity when the reaction is completed. Each cartridge contains 14 positions, 10 holes, and two optional heating positions. The reagents are prepared previously and can be loaded into the device directly, and the DNA can be extracted with high purity (Figure 3.4).

- 1000 μL of the rinse solution.

- 1500 X2 µL of wash solution.
- 1000 µL of deoxygenated water.
- 1000 µL f magnetic bead mixture.
- 1500 µL of binding solution.
- 1200 µL of lysis solution.

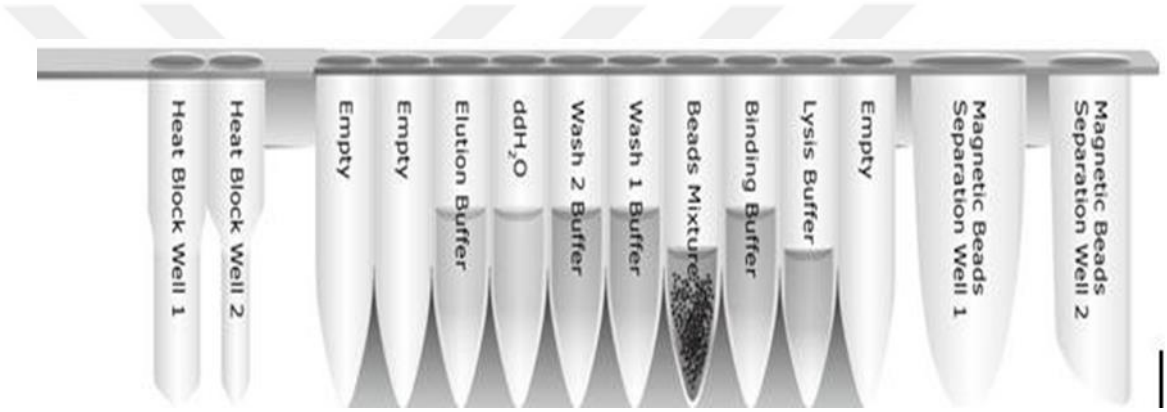

Figure 3.4 Cartridge components

Kit components preparation

1.1 mL of PK Storage Buffer was added to the Proteinase K tube and mixed by the vortex. The prepared Proteinase K was kept at 10 mg/mL at -20°C.

Procedure

- 20 µL of Proteinase K is added to 80 µL of the sample and then the sample tubes are placed in the place determined for them in Machine\Magnesia device.

- The cartridge containing the previously prepared solutions, the washing tubes, the set of tips, and the holder are placed in the places determined for them.

- The device is programmed at 104 programs at MagCore® and the reaction is completed after 76 minutes.

DNA electrophoresis

The diagnostic kit Kapa Express ladder from (Kapa, USA) was used for rapid electrophoresis to estimate the size and quality of DNA in agarose gel according to the method of (Tawfeeq 2015).

DNA loading and electrophoresis

20 µL of DNA was mixed with 5 µL of loading stain and then loaded into the gel carefully, after the electric power (100 V/cm) was turned on for 30 min. The DNA bundles were transferred from the cathode (-) to the anode (+). After the electrophoresis was completed, the DNA samples were observed by using a UV trans-illuminator stained with ethidium bromide stain using a wavelength of 320-302 nm.

3.19 Detection of Morphological Changes Using Acridine Orange\Propidium Iodide

This stain is used to dye DNA and detect changes that occur in cells due to programmed cell death (AO) stain is considered a positive ion with the ability to stain the nuclei of living and dead cells, as the nuclei of living cells appear in a bright green, spherical shape, and a healthy structure when examined under a fluorescent microscope by using a wavelength filter (blue), while PI stain enters the nuclei of dead cells in which a breakdown occurs in their membranes and which appear bright red color when examined under a fluorescence microscope by using a wavelength filter (green) (Alrawi 2017).

The solution used

- Acridine Orange stain solution at a concentration of 5 mg/mL.
- Propidium Iodide Stock stain Solution at a concentration of 3 mg/mL.
- PBS (pH 7.2).

Preparation of working solution

Add 100 µL of AO and 100 µL of PI to 1000 µL of PBS, mix well, and keep at room temperature for two weeks.

Procedure

- Grown the cancer cells in a 24-hole tissue culture plate, 2 mL of cell suspension is added to each hole and incubated for 24 hours at 37°C.
- When the cells adhere to the plate, the cells are treated with alkaloid extract at IC50 concentration and incubated for 24 hours.
- After the incubation period is over, the culture medium is discarded and 500 µL of AO/PI stain mixture is added to each hole and then incubated at 37°C for 20-30 minutes in the dark.
- After that, the cells are washed with PBS solution, and the washing process is repeated more than once until the excess stain is removed.
- Finally, 500 µL of PBS is added to each hole, and then the cells are visualized with an inverted fluorescence light microscope by using blue and green filters.

3.19 Statistical Analysis

The ANOVA analysis of the variance test,T test and F test and the arithmetic means were compared with the Tukey polynomial test (arithmetic mean±standard deviation M±SD) with a significant difference in the probability l.

4. RESULTS AND DISCUSSION

4.1 Preparation Biomass Productivity of Alga Culture Media for Chlorococcum humicola

Preparation of the Results The biomass productivity of C. humicola was evaluated in the same BBM used to maximize protein and chlorophyll synthesis in green algae (Sankar and Ramasubramanian, 2012). With a productivity of 73.8% mg/L/day, the alga is a promising prospect for producing a large amount of biomass and other byproducts, and it should be simple to put it through industrial testing.

Figure 4.1 Culture media for *Chlorococcum humicola*

A 25 mL was obtained from 1 gm of dry ground of alge and the extract was green.. There are synergistic effects of chemical compounds in all parts of the plant that are responsible for their biological properties such as, anticancer (Figure 4.2).

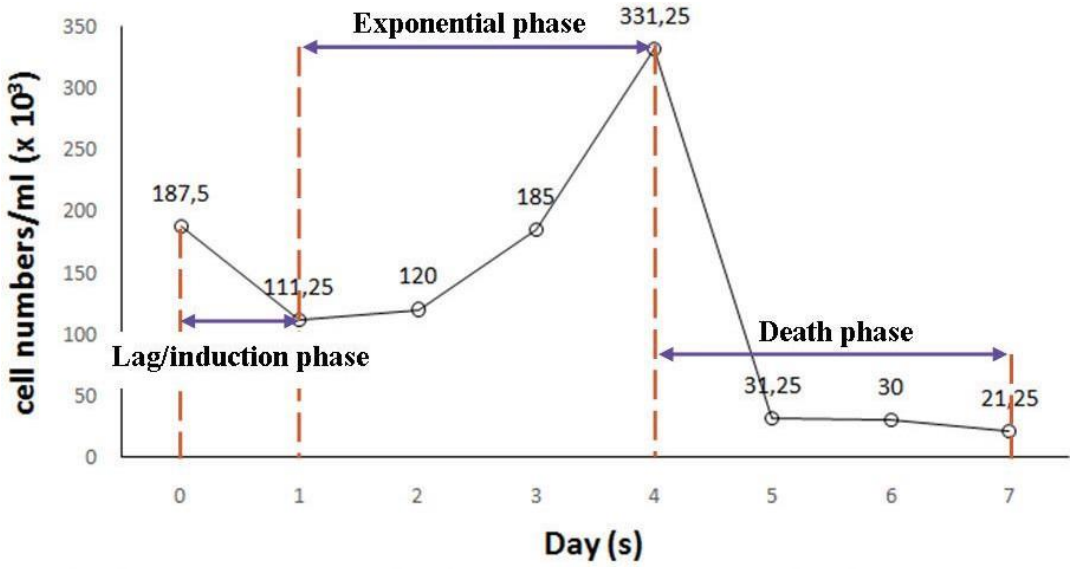

Figure 4.2 Growth curve of *Chlorococcum* spp.

4.2 Detection of Alkaloid Extracted from *Chlorococcum humicola*

After treatment of extract with Dragendroff's reagent, a Brown orange color appear, this indicates the presence of alkaloids. The alge drug analysis confirms the existence of an alkaloid with a comparable Rf value to other alkaloids in this genus as shown by Dragendroff's tests.

4.3 Effect of Alkaloid in Mitotic Index in Human Lymphocyte

Human lymphocytes were treated with five concentrations of crude alkaloid extract form alge *Chlorococcum humicola*. This test included treatment of lymphocytes with alkaloid extract in the presence of PHA.

4.3.1 Effect of alkaloid as a stimulant for lymphocyte division

The crude alkaloid extract of *Chlorococcum humicola*.. was used by adding it instead of the PHA by using five concentrations ranging between 50, 100, 200, 400, and 800 µg/mL. The results showed that there was no stimulation for lymphocyte division for all

concentrations used. This is evidence that the extracts can't stimulate lymphocytes to divide.

4.3.2 The anti-division effect of crude alkaloid extract *Chlorococcum humicola* on a lymphocyte

Test the efficacy of alkaloid extract in stopping lymphocyte division, the treatment led to an increase in the rate of lymphocyte division as the concentration increased, as shown in Table 4.1. The percentage of suspended cells stopped in the metaphase was 1.76, 1.90, 2.55, 3.99, 4.40 at concentrations 50, 100, 200, 400, and 800 µg/mL respectively, while the percentage of stopped cells in the control was 4.50. There was no significant difference between the two concentrations 400 and 800 µg/mL respectively and control, and the difference was significant between the percentages of suspended cells at other concentrations. When cells were treated with a concentration of 50 µg/mL, the percentage of suspended cells in the metaphase phase was 16.50 %, and this percentage increased to

43.44 % at the concentration was 100 µg/mL and the increase continued until the percentage reached 95.17% at the concentration of 800 µg/mL as shown in Figure 4.3, Figure 4.4 and Figure 4.5.

Table 4.1 Effect of alkaloid extract in mitotic index on human lymphocyte in 24 hours of exposure at 37℃ and compared with colchicine

Con. µg/mL	Cell ratio in metaphase inhibition ratio ± Standard deviation	A percentage from control %
Control(Colchicine)	4.50 a ± 0.01	-
50	1.76 d ± 0.14	16.50
100	1.90 c ± 0.11	43.44
200	2.55 b ± 0.08	65.48
400	3.99 a ± 0.13	90.67
800	4.40 a ± 0.25	98.30

The different letters in the same column indicate that there are statistical differences at the level of (0.05≥P)

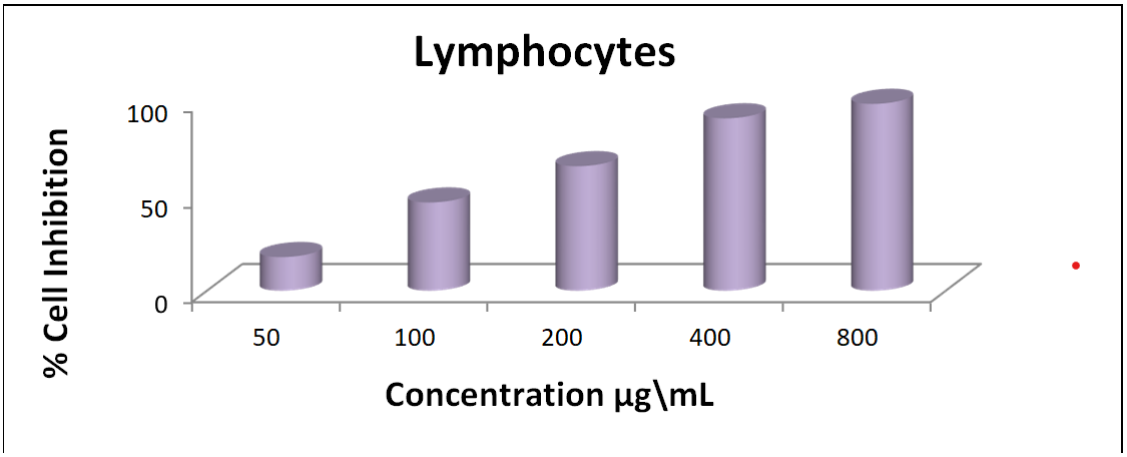

Figure 4.3 Effect of alkaloid extract in mitotic index on human lymphocyte in 24 hours of exposure at 37℃

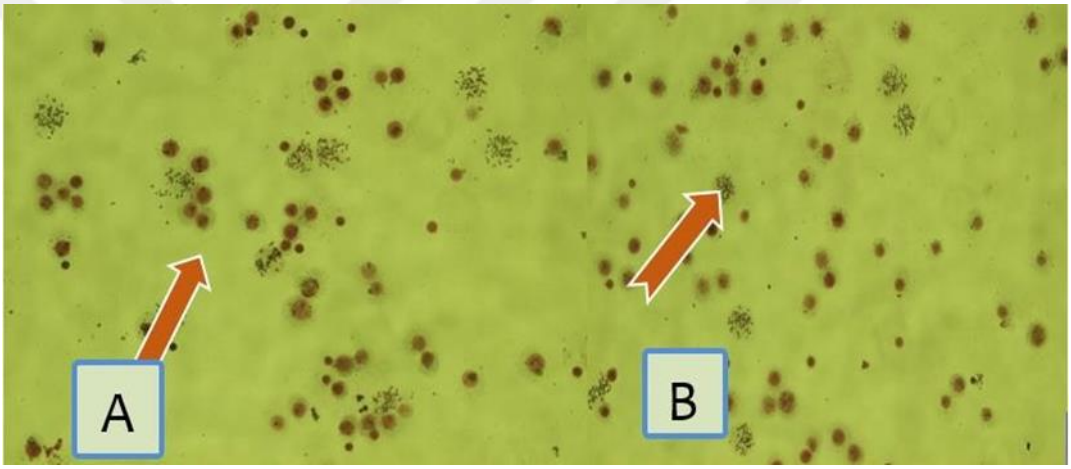

Figure 4.4 Comparison between Lymphocytes that were treated and untreated with alkaloid extract at a concentration of 800 µg/mL for 24 hours of exposure at 37°C (x20) by using Crystal Violate stain. (A) representing control lymphocytes. (B) representing treated ly

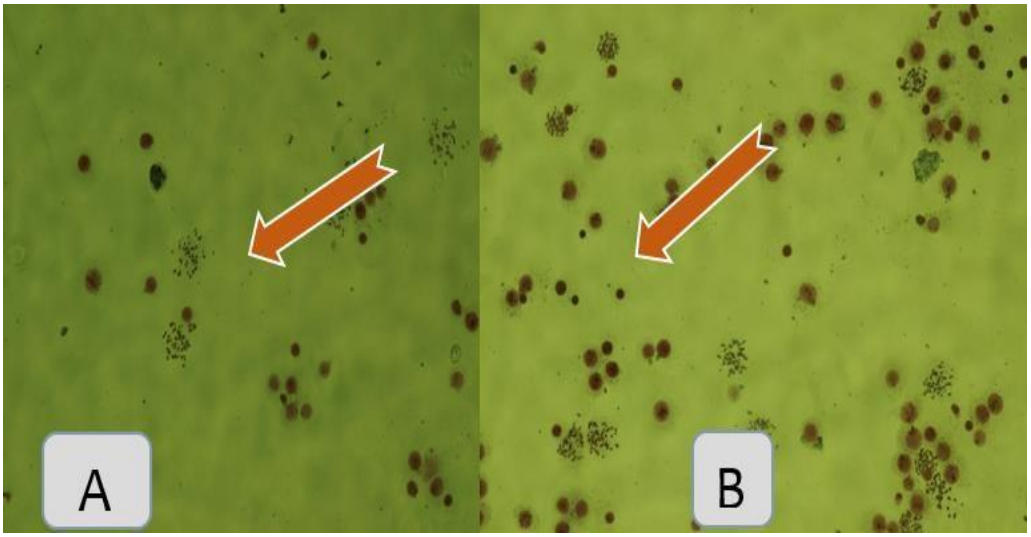

Figure 4.5 Comparison between Lymphocytes that were treated and untreated alkaloid extract at a concentration of 800 µg/mL for 24 hours exposure at 37°C (100x) by using Crystal Violate stain. (A) representing control lymphocytes. (B) representing treated lymphocytes with alkaloid extract at a concentration of 800 µg/mL and showing

From these results, we can be inferred that the alkaloid extract of *Chlorococcum humicola*

L. can stop the dividing of lymphocytes in metaphase. The extract of alkaloids extracted from *Chlorococcum humicola* decreased the mitotic index of human lymphocytes from 4.6% to 1.4% when the concentration is increased from 5µg\mL to 200µg\mL. The large drop of MI could be due to the blocking of G2 events, which inhibits the cell from entering mitotic division or it might be the result of ATP levels having dropped (Ahmad *et al.* 2019).

4.4 Effect of Alkaloid Extract on Cancer and Normal Cell Lines

To test the effect of the alkaloid extract's ability in the growth of cancerous tumors, the test was conducted on two cancer lines (MCF7, Hela) and one normal cell line (RD). Cancer cell lines were treated with five concentrations and three replicates for each concentration of the plant alkaloid extract for 24 hours at a temperature of 37 by using different concentrations that are 50, 100, 200, 400, and 800 µg/mL, and the cytotoxicity

test was adopted to determine the effect of the concentrations of the extract on the growth of the cells, in a term of the percentage of the rate of inhibition of growth.

Table 4.2 showed that the crude alkaloid extract had an inhibitory effect on the growth of cancer cells of the MCF7 line, starting with a concentration of 50µg/mL, as the percentage of inhibition was 30.05%, and this percentage increased to 40.23%, 79.56 %, 80.40 %, and 90.45 % for the concentrations 100, 200, 400, and 800 µg/mL, respectively. A significant difference was observed between concentrations as shown in Figure 4.6.

Table 4.3 showed that the alkaloid extract had an inhibitory effect on the Hela cancer cell line that started with a concentration of 50µg/mL, as the percentage of inhibition reached 20.88% and increased to 50.58%, 70.44%, 78.91%, 95.39% for the concentrations 100, 200, 400, and 800 µg/mL, respectively. No significant difference was observed (Figure 4.7).

Table 4.4 showed that the alkaloid extract had a little inhibiting effect on the normal cell line (RD). At concentrations 50, 100, and 200 µg/mL, the percentage of inhibition was 0% and increased to 9.9 % and 10.11% for concentrations 400 and 800 µg/mL, respectively. No significant difference was observed between the concentrations 50, 100, and 200 µg/mL as shown in Figure 4.8. According to the statistical results, it was found that there was a significant difference when comparing the effect of the alkaloid extract on cancerous lines after using the IC50 concentration, which is for the MCF7 line (300), for the Hela line (100), and the normal line RD (400) as shown in the Table 4.5, Figure 4.9, Figure 4.10 and Figure 4.11.

Table 4.2 Inhibition percentage in the MCF7 cancer cell line by the effect of different concentrations of nicotine for 24 Hours of exposure at 37°C

Con. µg /mL	Inhibition ratio ± Standard deviation
50	30.07 e ± 2.6
100	40.23 d ± 3.1
200	79.56 c ± 4.2
400	80.40 b ± 7.9
800	99.45 a ± 8.5

The different letters in the same column indicate that there are statistical differences at the level of (0.05≥P)

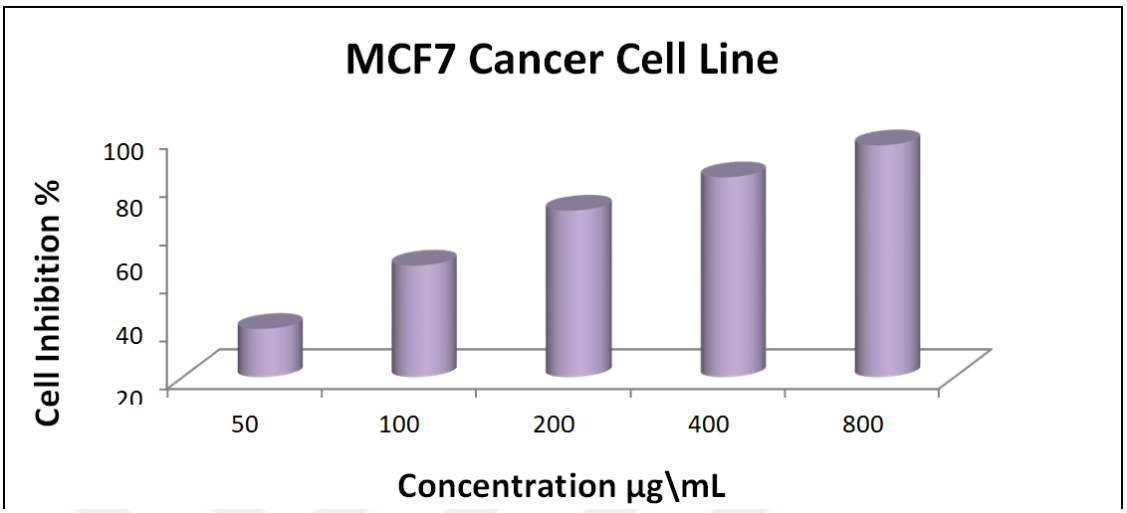

Figure 4.6 Inhibition percentage in the MCF7 cancer cell line by the effect of different concentrations of nicotine for a 24 hours of exposure at 37°C

Table 4.3 Inhibition percentage in the Hela cancer cell line by the effect of different concentrations of for a period of exposure for 24 hours at 37°C

Con. µg /mL	Inhibition ratio ± Standard deviation
50	22.19 d ± 2.6
100	49.98 c ± 7.1
200	61.11 b ± 8.3
400	88.92 a ± 8.6
800	90.31 a ± 7.1

The different letters in the same column indicate that there are statistical differences at the level of (0.05≥P)

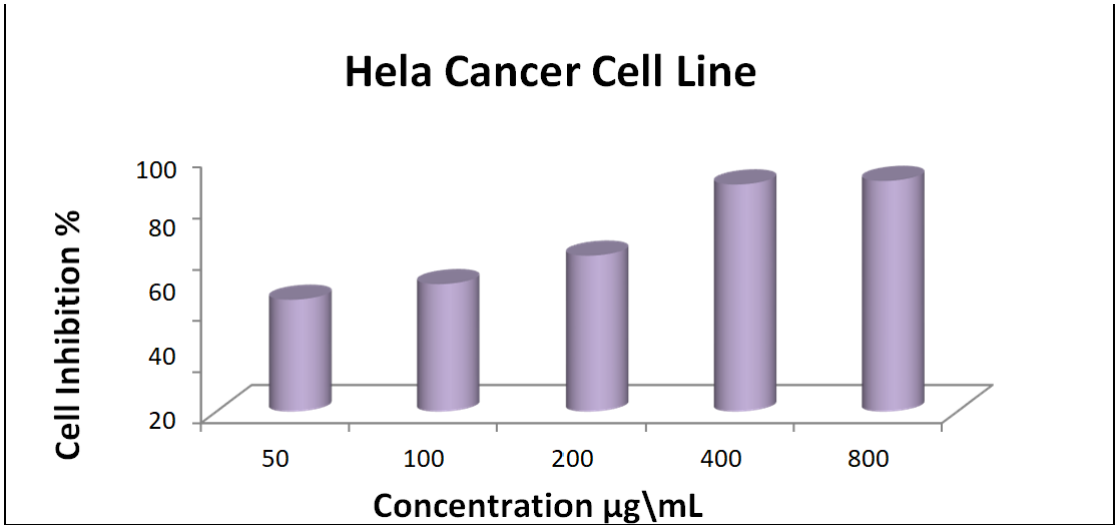

Figure 4.7 Inhibition percentage in the Hela cancer cell line by the effect of different concentrations of for a 24 hours of exposure at 37°C

Table 4.4 Inhibition percentage in the RD normal cell line by the effect of different concentrations of for 24 hours of exposure at 37°C

Con. µg /mL	Inhibition ratio ± Standard deviation
50	0 c
100	0 c
200	0 c
400	9.9 b ± 2.1
800	10.11 ± 2.4

The different letters in the same column indicate that there are statistical differences at the level of (0.05≥P)

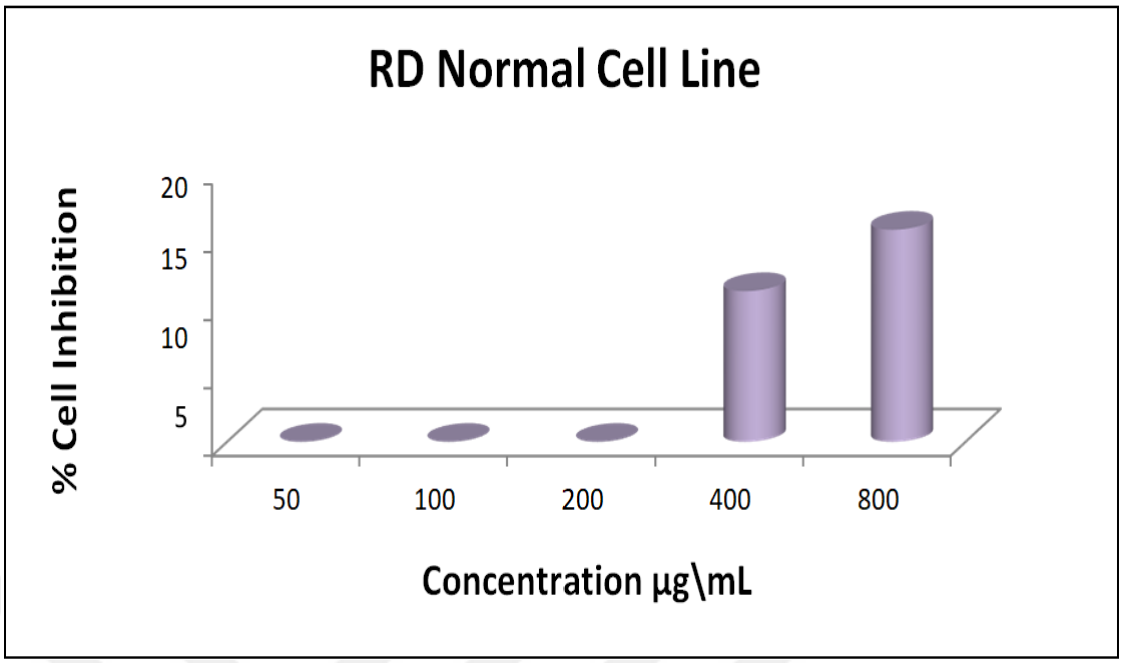

Figure 4.8 Inhibition percentage in the RD normal cell line by the effect of different concentrations of for 24 hours of exposure at 37°C

Table 4.5 IC50 concentration values in the cancer cell lines MCF7, Hella, and the normal cell line RD

Cell Line	IC50 con. µg/mL
MCF7	300
Hela	100
RD	400

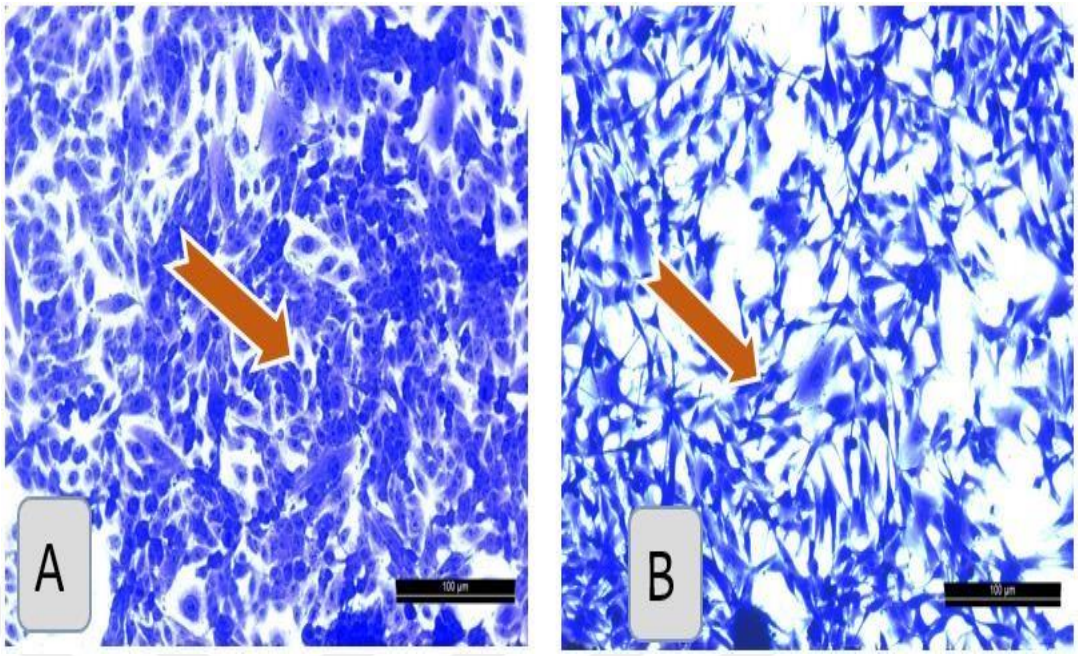

Figure 4.9 Comparison between cells of MCF7 line that treated and untreated with alkaloid extract at a concentration of 800 µg/mL for 24 hours exposure at 37° C (x100) by using Crystal Violate stain. (A)MCF7 cancer cell line representing control and showing dense cells. (B) MCF7 cell line treated with alkaloid extract at a concentration of 800 µg/mL and showing dead cells and voids between cells

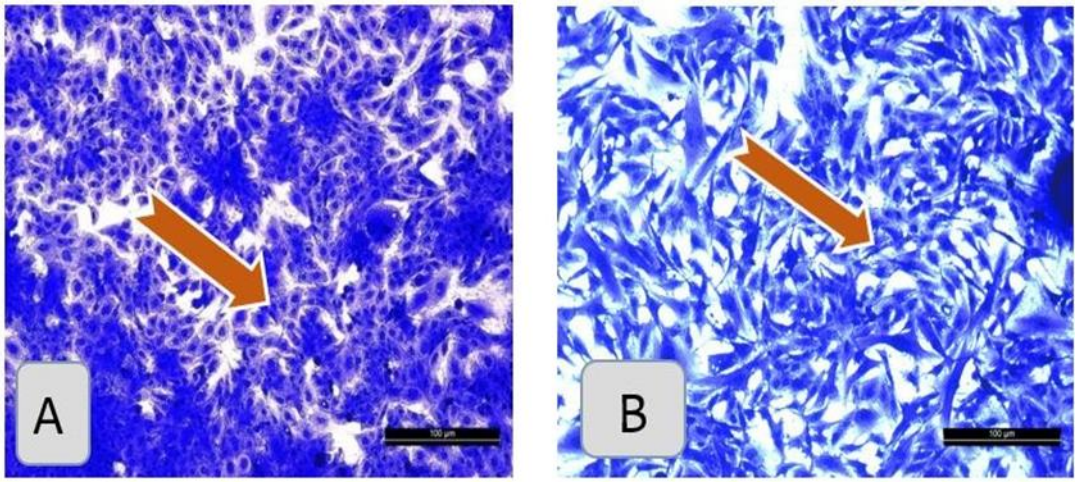

Figure 4.10 Comparison between cells of Hela line that treated and untreated with alkaloid extract at a concentration of 800 µg/mL for 24 hours exposure at 37°C (x100) by using Crystal Violate stain. (A) Hela cancer cell line representing control and showing dense cells. (B) Hela cell line treated with alkaloid extract at a concentration of 800 µg/mL

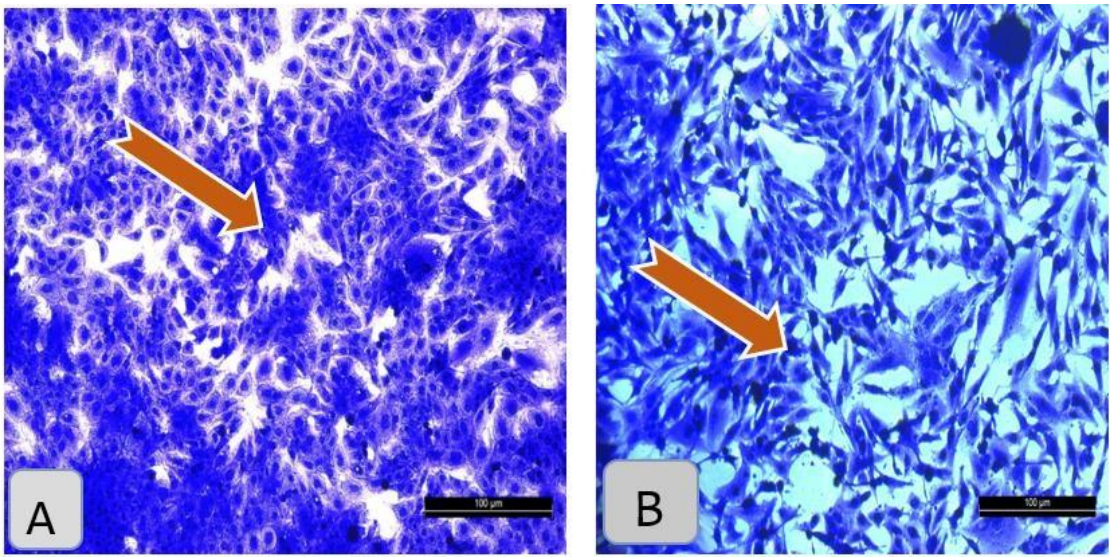

Figure 4.11 Comparison between cells of RD line that treated and untreated with alkaloid extract at a concentration of 800 µg/mL for 24 hours exposure at 37°C (x100) by using Crystal Violate stain. (A) RD cancer cell line representing control and showing dense cells. (B) RD cell line treated with alkaloid extract at a concentration of 800 µg/mL and showing dead cells and voids between cells

The results of this study reinforced the findings of many researchers about plant extracts having anti-cancer activity. This effectiveness depends on the type and concentration of extract, in addition to the sensitivity of cancer cells (Al-Ansari *et al.* 2021). The results showed the toxic effects of the alkaloid extract of *Chlorococcum humicola L.* on the growth of cancer cell lines(MCF7 and Hela) for all the concentrations that used as shown in Table 4.3 and Table 4.4. The results also showed an effect of the alkaloid extract on the normal cell line (RD) at higher concentrations, but at lower rates than in the cancer cell lines, as shown in the Table 4.5. The inhibitory effect of the extract on cancer cells is that it contains many chemical compounds, especially alkaloid, which is effective in inhibiting or stopping the growth of cancer cells. The study of (Al-Lahham *et al.* 2020) showed that the aqueous and methanol extracts of *Chlorococcum humicola L.* roots have an anti-proliferative effect on HeLa cervical adenocarcinoma. Also, the flavonoid extract of has a potent cytotoxic effect on MCF7 human breast cancer cells.

It was discovered that the alkaloid extract of Coptis Chinensis Franch inhibits the multiplication of breast and liver cancer cells by halting the cell cycle and producing

stress in the endoplasmic reticulum, resulting in cancer cell metastasis inhibition. Another

study looked at the effects of alkaloid extract on the development of lung cancer (A549), breast cancer (MCF-7), hepatocellular carcinoma (HepG-2), and normal human fetal lung fibroblast (WI-38) cell lines by using ether, chloroform, and aqueous extracts of *Cynanchum acutum L.* seeds (Youssef *et al.* 2019).

4.5 Extraction of RNA and its Conversion to Complementary DNA from Cancer Cell Lines

Total RNA was extracted from cells of the MCF7 cancer cell line that were treated and untreated with alkaloid extract at IC50 (300 μg/mL) for 24 hours, as well as from cells of the Hela cancer cell line that were treated and untreated with alkaloid extract at IC50 (100 μg/mL) for 24 hours. Also from cells of RD normal cell line that treated and untreated with alkaloid at IC50 (400 μg/mL). The concentration of RNA from each cancer cell line that transferred to cDNA was determined by using a Spectrophotometer Nano Drop, and it varied from 1290 to 1310 ng/microliter, with purity between 1.70 and 1.80.

4.6 Effect of Alkaloid Extract on Gene Expression of Heat Shock Proteins Genes (Hsp70 and HIF) in Cancer Cell Lines

Syber green dye, a fluorescent dye that can differentiate any DNA even complementary DNA, was used in RT-PCR to measure gene expression. The expression of genes that code for heat shock proteins in cancer cell lines is suppressed after 24 hours of treatment with alkaloid extract at IC50 (300, 100). Table 4.6 shows that compared to the control group, treated cells expressed less of the genes encoding heat shock proteins Hsp70 and HIF in the MCF7cancer cell line (Figure 4.12), and more of the genes encoding these proteins in the Helacancer cell line (Figure 4.13).

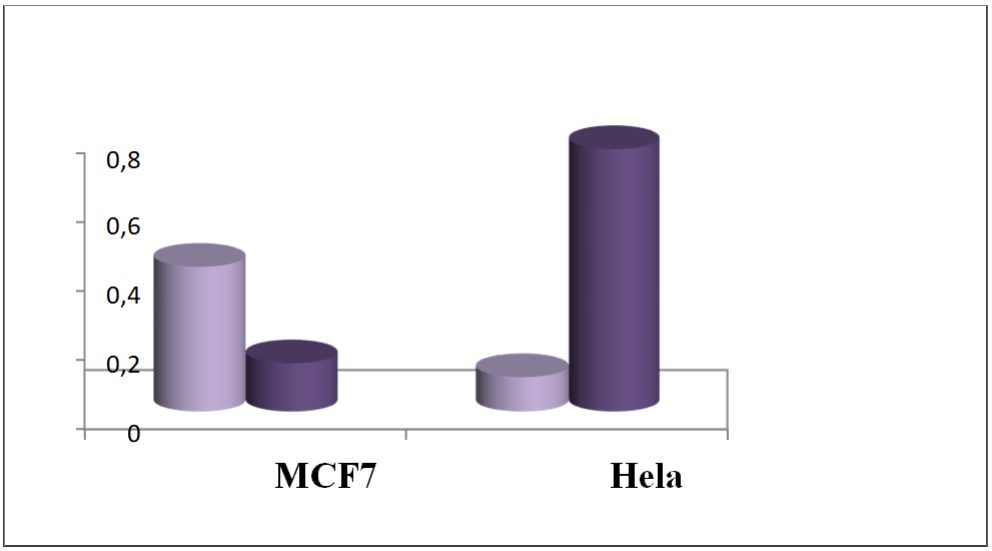

Figure 4.12 Change in the gene expression of heat shock proteins genes (Hsp70, HIF) in cancer cell lines

Table 4.6 Change in the gene expression of heat shock proteins genes (Hsp70, HIF) in cancer cell lines

Gene type	MCF7 Gene Expression	Hela Gene Expression
Hsp70	0.45	0.11
HIF	0.18	0.77

There are several ways in which heat shock proteins prevent cell death from occurring. Overexpression of heat shock protein 70 (HSP70) has been linked to a variety of cancers. Since HSP70 inhibits apoptosis, it has been proposed as a therapeutic target for cancer (Pirali *et al.* 2020). Lung cancer cell lines are unable to develop or proliferate when Hsp70 is inhibited (Mittal and Rajala, 2020). Many kinds of cancer benefit from HIF overexpression, which promotes tumor growth and spread through many routes (Rashid *et al.* 2021).

4.7 Effect of Alkaloid Extract on Gene Expression of Programmed Cell Death Genes (P53, BAX) in Normal and Cancer Cell Lines

Nicotine alkaloid extract at IC50 (300,100,400) for 24 hours boosted gene expression of the BAX expressing gene in cancer (MCF7, Hela) and normal (RD) cell lines. The

expression of the Caspase-9 encoding gene decreased by 1.89, 2.70, and 3.41 percentage points, respectively, in MCF7, Hela, and RD cell lines after treatment with alkaloid extract compared to the control group. Changes in the expression of the genes encoding p53 and BAX were 0.14 and 0.48 in the MCF7 cell line and 0.16 and 0.52 in the Hela cell line, respectively. Table 4.7 and Figure 4.13 reveal that for the RD cell line, these numbers were 1.68 and 0.42, respectively.

Table 4.7 Change in the gene expression of programmed cell death genes (P53, BAX) in cancer cell lines

Gene Type	MCF7 Gene Expression	Hela Gene Expression	RD Gene Expression
P53	0.14	0.16	1.68
BAX	0.48	0.52	0.42

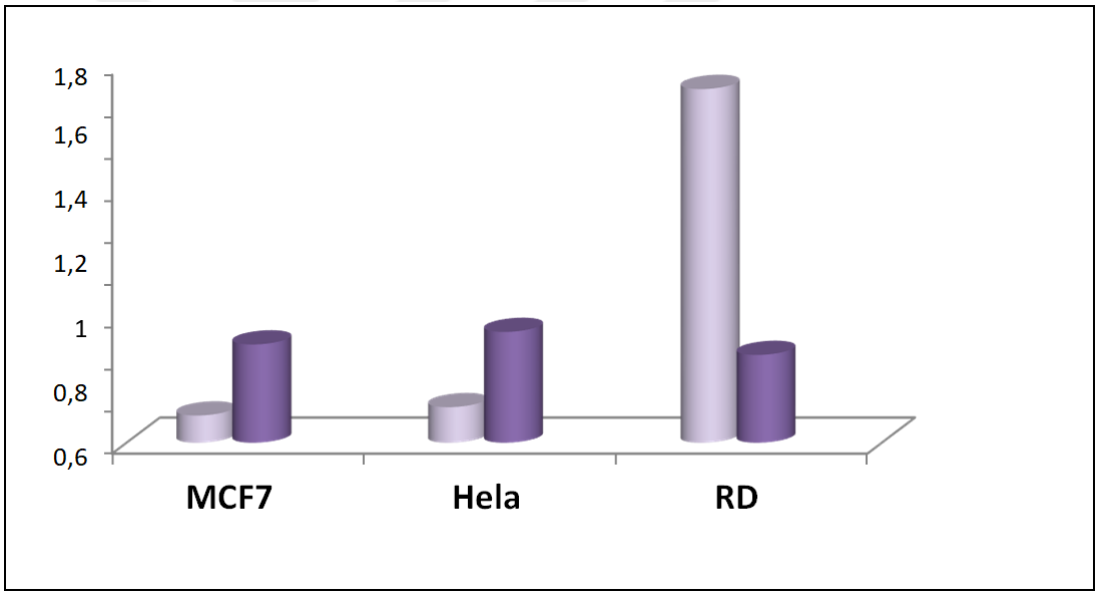

Figure 4.13 Change in the gene expression of programmed cell death genes (P53, BAX) in cancer cell lines

Apoptosis is an essential part of eukaryotic development and the preservation of organismal homeostasis, and it has been preserved throughout evolution. This mechanism is controlled by the BCL-2 protein family, which has both pro-apoptotic and pro-survival members (Singh *et al.* 2007). Many studies show that plant extracts can induce cancer cells to programmed cell death. The study indicated by (Pathiranage *et al.* 2020) showed that the organic solvent extracts of the polyherbal mixture containing

seeds of Nigella sativa, roots of Hemidesmus indicus, and rhizomes of Smilax glabra have a significant up-regulation of the Bax gene in the NCI-H292 cancer cell line. Also, the water extract of the Hibiscus sabdariffa leaf belonging to the Malvaceae family can induce apoptosis in the prostate (LNCaP cells) cancer cell line by increasing the expression of BAX and cytochrome-C of cytoplasm and then activating Caspase-9 (Rajabi *et al.* 2021).

4.8 Effect of Alkaloid Extract on DNA Fragmentation

In this study the DNA fragmentation was detected by extracting DNA from MCF7 and Hela cells treated with alkaloid extract at IC50 for 24 hours, then the DNA fragmentation was examined by using agarose gel electrophoresis. Figure 4.14 shows a significant indicator of programmed cell death (DNA fragmentation) in the cancer lines treated with the crude alkaloid extract compared to the control group. The results showed that significant damages occurred, represented by a break in the DNA in the agarose gel of the treated cells, while the control group showed minimal damage.

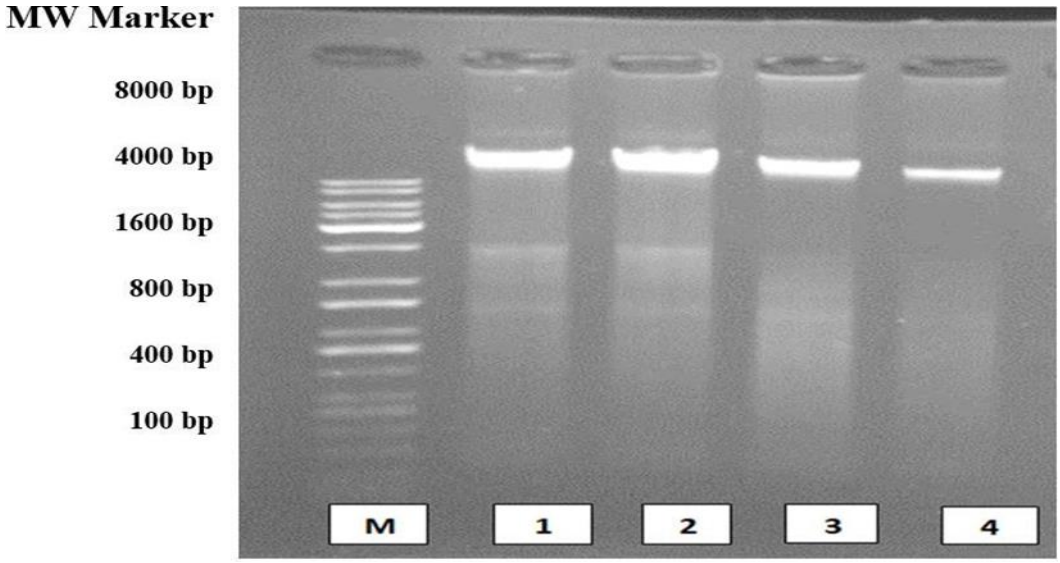

Figure 4.14 Effect of alkaloid extract on DNA fragmentation by using agarose gel electrophoresis M: molecular weight marker, 1: a control group of MCF7, 2: a control group of Hela, 3: a treated group of MCF7, 4: a treated group of Hela

4.9 Detection of Morphological Changes in Cell Lines by using Acridine Orange\Propidium Iodide (AO\PI)

When cancer and normal cells were exposed to alkaloid extract at IC50 for 24 hours, their impact on MCF7, Hela, and RD cell lines was seen by inducing them to die when inspected by fluorescence microscopy as shown in Figure 4.15, Figure 4.16 and Figure 4.17, respectively, by using (AO\PI) stain. Through direct observation or through using fluorescence microscopy, which exposes normal cells and cells that have died due to programmed cell death or necrosis. This approach was used to analyze morphological alterations (such as nuclear modifications and the creation of apoptotic bodies) and to distinguish between live and dead cells that had undergone programmed cell death.

Due to the penetration of (AO) stain into the cell membrane and DNA bundles, all nuclei in living cells in the control group (untreated cells) looked green in color, with a normal spherical shape and regular chromatin. But the nuclei of cells that had undergone programmed cell death appeared red with fragmented nuclei and compacted chromatin.

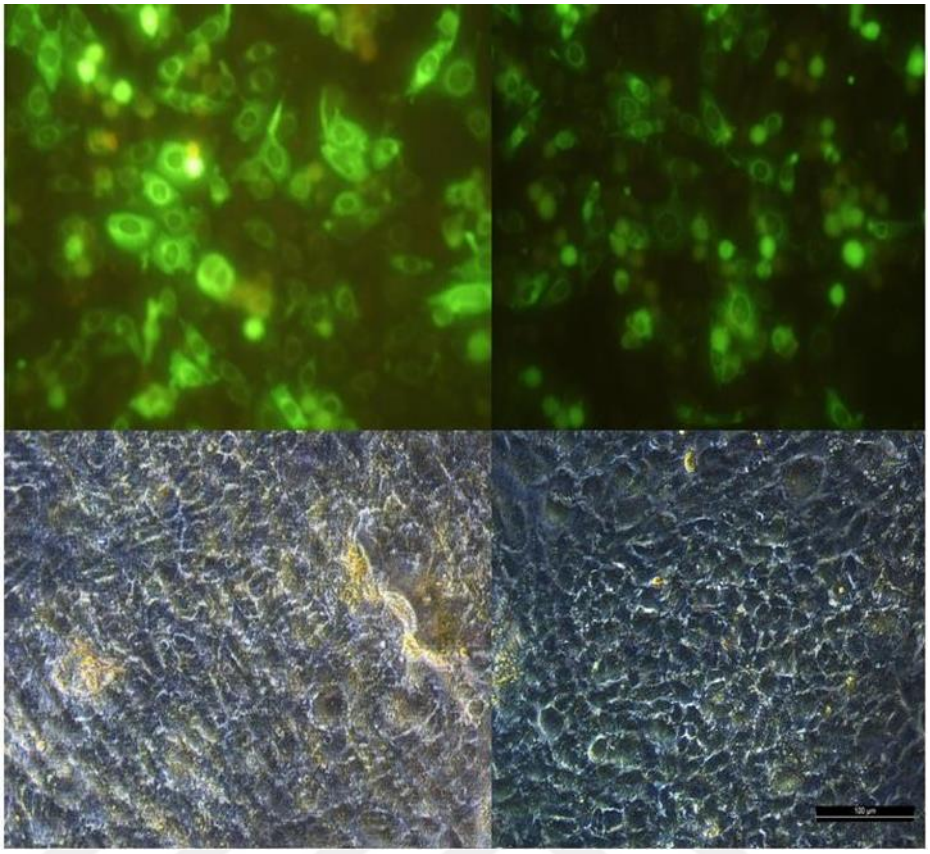

Figure 4.15 Detection of morphological changes of programmer cell death by stain in MCF7 cancer cell line that treated with alkaloid extract at IC50 for 24 hours of exposure (100X). A) representing untreated cells with alkaloid extract (Control Group). B) representing cells that treated with alkaloid extract. white raw refer to living cells, while green raw refer to dying cells

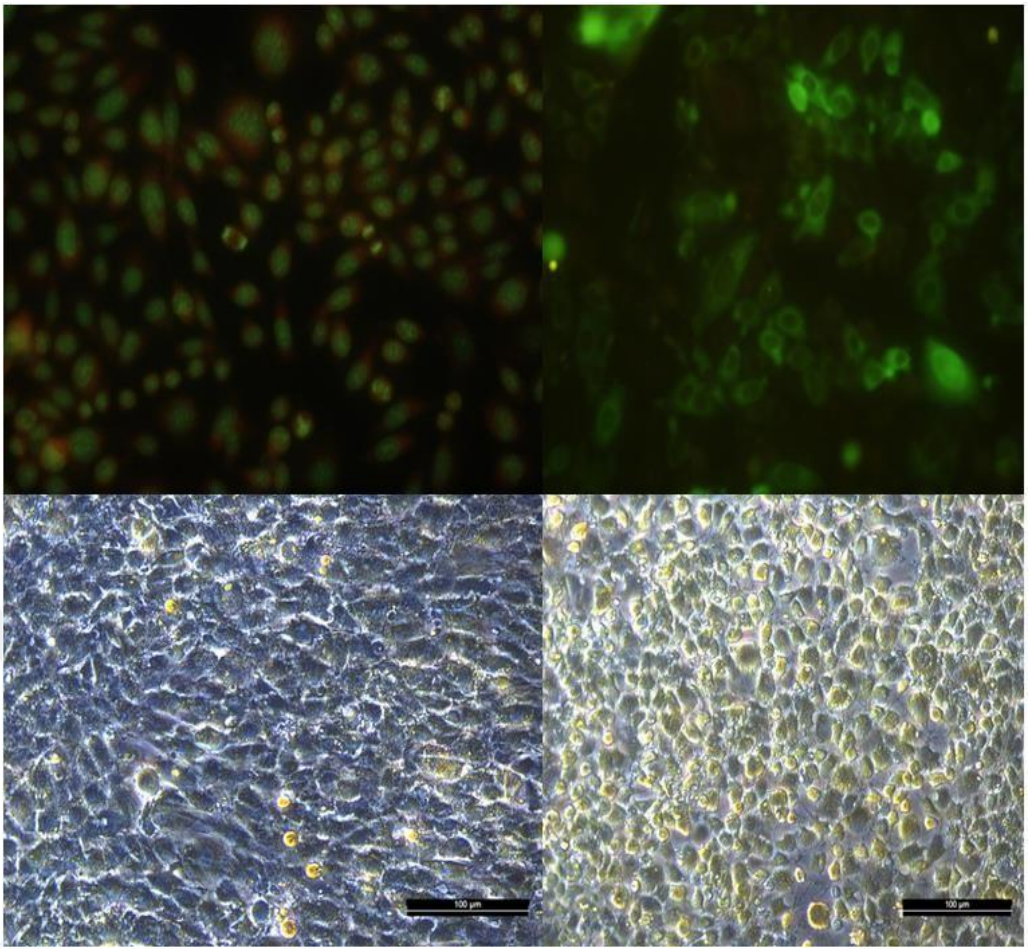

Figure 4.16 Detection of morphological changes of programmer cell death by stain in Hela cancer cell line that treated with alkaloid extract at IC50 for 24 hours of Exposure (100X). A) representing untreated cells with alkaloid extract (Control Group). B) representing cells that treated with alkaloid extract. white raw refer to living cells, while green raw refer to dying cells

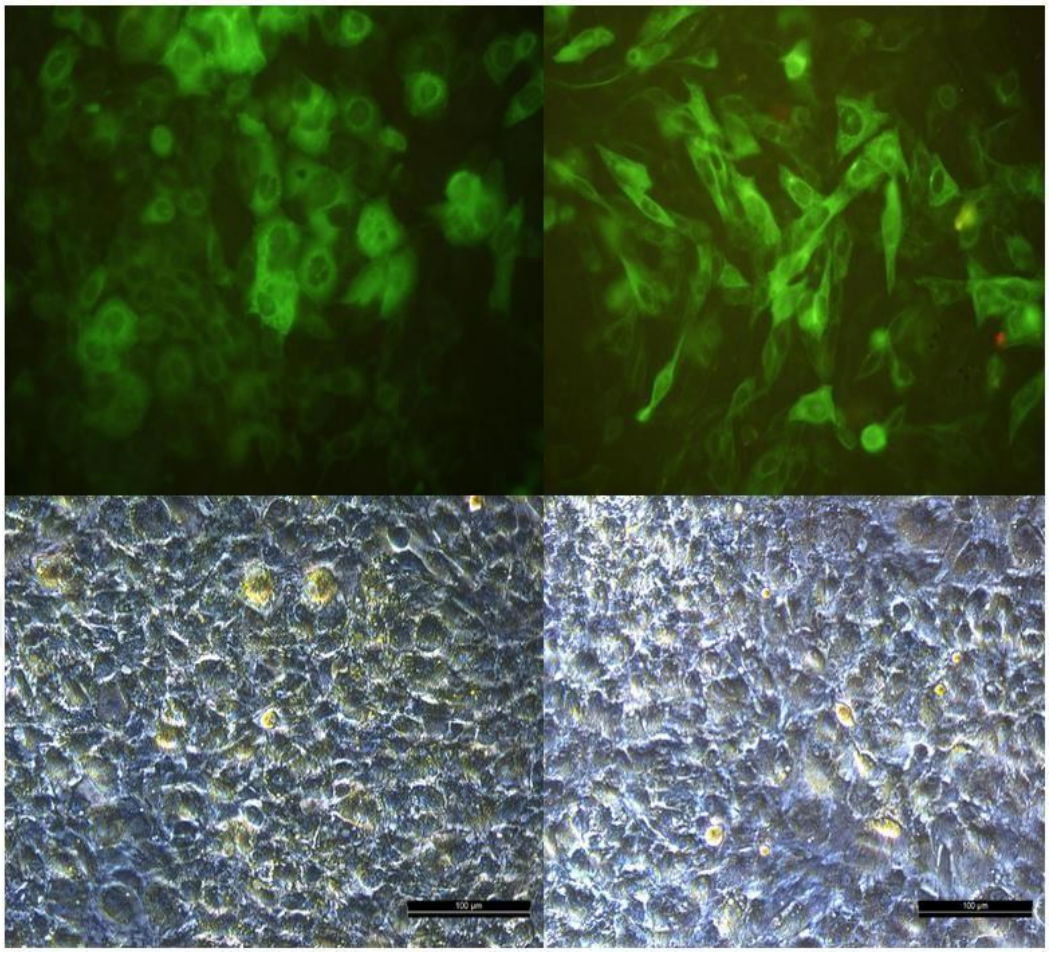

Figure 4.17 Detection of morphological changes of programmer cell death by stain in RD cancer cell line that treated with alkaloid extract at IC50 for 24 hours of Exposure (100X). A) representing untreated cells with alkaloid extract (Control Group). B) representing cells that treated with nicotine alkaloid extract. white raw refer to living cells, while green raw refer to dying cells

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

It could be concluded from the present study that:

- The extract of alkaloids from Chlorococcum humicola. contained a high percentage from him.

- The alkaloids extract from Chlorococcum humicola has an anti-division effect on lymphocytes.

- The alka; oids extract has an inhibitory effect on the growth of (MCSF7 and Hela) cancer cell lines.

- The alkaloids extract has an inhibitory effect on the expression of heat shock proteins encoding genes (Hsp60 and Hsp70) in cancer cell lines and increased the expression of programmed cell death encoding genes (Caspase-8, Caspase 9) in cancer (MCSF7 and HHela) and normal (RD) cell lines.

- The alkaloids extract can cause the death of cancer cells by inducing of programmed cell death process through many biological activities.

5.2 Recommendation

- Testing the antioxidant activity of the alkaloids extract.

- Studying the effect of alkaloids extract on the gene expression in some bacterial and yeast species.

- Studying the effect of alkaloids extract on other cancer cell lines, especially lung cancer.

- Studying the effect of alkaloids extract on the expression of other heat shock proteins encoding genes.

- Studying the effect of alkaloids extract on the expression of other programmed cell death encoding genes..

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