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Effect of Crude Alkaloid Extract of *Cladophora glomerata* (L.) kutz. On Gene Expression and Cell Death

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Abstract

The objective of this study was to determine the physiological effects of the crude alkaloid extract from *Cladophora glomerata* at the half inhibitory concentration (300, 100, and 1000) µg /ml on the growth rate in the gene expression of the heat shock proteins (Hsp90, Hsp70, Hsp60), as well as the cell death genes (Caspase-8, P53, and Bcl2) of the cancer cell lines Hepatocellular carcinoma (HepG2), human cervical cancer (Hela), and mice embryo fibroblast (MEF). In addition to monitoring morphological changes using real-time polymerase chain reaction for gene analysis using Kapa syber green dye, and observing morphological changes using Acridine Orange / Propidium Iodid dye that examined using a fluorescence optical microscope. The results of this study showed that treating cancer cell lines with the crude alkaloid extract at half inhibitory concentration effectively suppresses the gene expression of heat shock protein that encode genes and increases the expression of programmed cell death genes. We conclude that *Cladophora glomerata* extract plays a major role in inhibiting cancer cells (HepG2 and Hela cell lines) through regulating heat shock protein genes (Hsp90, HSp70, HSp60) and apoptosis genes (P53, Bcl2, and Caspase- 8) which have a major role in cancer.

Keywords; *Cladophora glomerata*, cell lines, apoptosis genes, and heat shock proteins genes.

Introduction

Cladophora glomerata is a green, branched filamentous algae widespread in freshwater environments [1]. *C. glomerata* possesses several bioactive features and is widely regarded as an important algae in the fields of medicine and pharmaceuticals, alongside its other applications,Many researchers have proven its medical effectiveness due to its antibacterial, antifungal, antiparasitic and anti-inflammatory effects [2]. In addition, it exhibits efficacy in combating diabetes, as an anti-ulcer, antioxidant, and anti-cancer [3]. *C. glomerata* contains many biologically active molecules such as alkaloids and flavonoids [4].

Chemotherapy continues to be the treatment of choice for cancer patients despite the availability of other medical options. The number of the mucous membranes of different organs can be affected by the medications that are used in chemotherapy, despite the fact that even thoughdrugs can destroy rapidly growing malignant tissues successfully, Some of the adverse reactions that can occur in cancer patients as a result of the treatment include anaphylaxis, toxicity to the liver, nausea, vomiting, diarrhea, alopecia, anorexia, inflammation in mucous membranes, and asthenia, which are just some of the side effects that can be observed [5]. Antioxidant supplements are frequently prescribed as a means of compensating for these negative effects. Furthermore, these supplements have the potential to alleviate side effects without compromising the effectiveness of the treatment [6]. Those who have survived cancer frequently take supplements of vitamins or minerals, natural products derived from plants, or herbal medicines in order to ease the adverse effects that are associated with their treatment[7]. The researchers have deduced that the methanol extracts derived from the algae *C. glomerata* possess remarkable anticancer and antioxidant properties. Furthermore, it has been observed that *C. glomerata* plays a significant role in the treatment of colon and breast cancer [8]. Research has demonstrated that the ethanolic extracts of *C. glomerata* exhibit notable anti-tumor effects in KB human oral cancer cell lines [9].

Apoptosis is a natural cellular process of programmed cell death, presents significant promise as a focal point for the development of anticancer therapies. Both the and extrinsic pathways utilize caspases to carry out apoptosis through the process of cleaving many proteins. In cancer,

the process of programmed cell death, known as apoptosis, is usually hindered through many mechanisms, such as the excessive production of proteins that prevent apoptosis and the insufficient production of proteins that promote apoptosis [10]. Several of these alterations result in inherent resistance to the predominant form of cancer treatment, chemotherapy. Plant-derived chemicals with anticancer activity show promise as novel therapeutics by activating the apoptotic pathway [11].

Subsequently, it became increasingly evident that heat shock proteins (HSPs) had a significant impact on the development of cancer and the advancement of malignancy through their regulation of angiogenesis, cell proliferation, apoptosis, migration, invasion, and metastasis. Consequently, HSPs have garnered growing interest as biomarkers for cancer diagnosis and as targets for cancer treatment [12]. Activation of heat shock factor (HSF) and elevated levels of heat shock proteins (HSPs) have been linked to early detection, prognostic prediction, postoperative surveillance, and anticipation of drug resistance in different cancer types. These findings suggest that HSF and HSPs have promise as clinical biomarkers. In addition, HSPs also control metabolic pathways and contribute to cancer metabolism, hence creating additional possibilities for anticancer treatments[13].

Due to lack of researches about the effects of *C. glomerata* extract on gene expression and programmed cell death genes in cancer cell lines, our study aim to detecte the effect of *C. glomerata* algae extract on gene expression of heat shock protein genes (Hsp90, HSp70, and HSp60) and programmed cell death genes (P53, Bcl2, and Caspase 8) in cancer cell lines HepG2, Hela, and MEF

Materials and Methods

Collection of algae samples

The current study was conducted during the fall of 2022. Algae samples were collected using a plankton collection net with holes with a diameter of 20 microns from Hassan Al-Hamoud stream near the entrance of the College of Engineering, University of Diyala. Its coordinates according to longitude and latitude were N: 33.77633048127538, E: 44.59493871588987.

Samples were stored in sterile, carefully sealed plastic containers, and the date and place of collection of each sample were independently verified.

Morphological characterization

The samples were inspected using a light microscope with magnifications of 40x and 100x, to identify the algae obtained throughout the modeling process. To classify them, determine the target genus, compare them to the forms in the deliberate classification books, and classify algae, use the approved classification source [14].

Preparation of the extract from *Cladophora glomerata*

Crude alkaloids were extracted from the moss *C. glomerata* as described by Cannell (1988)[15]. Using a blender, the dried *C. glomerata* moss was ground into a powder. Then, a Soxhlet apparatus was used to extract the crude alkaloid in the moss according to the method of Cannell (1988) (14). Alkaloids in the extract were detected using Dragendorff's reagent. The presence of alkaloid was shown by the appearance of a brown-orange color when the extract was exposing to the reagent. In this investigation, concentrations of 15.1, 31.2, 62.5,125, 250, 500, and 1000 µg/ml were employed.

The utilized cancer cell lines

Cell lines maintenance and culture

The utilized Cancer cell lines in this study were obtained from the Iraqi Centre for Cancer Research and Medical Genetics. The production of these items utilized the methodology outlined by Fresheny (2015)[16]. The cells used in this study include HepG2, Hela, and MEF. The extract was added to a 25 cm^2 tissue culture plate together with a trypsin-versene solution. Twenty milliliters of RPMI-1640 were combined with the Foetal Calf Serum (FCS) and thoroughly mixed. Subsequently, 0.2 cc of the mixture was introduced into each of the 96 wells of a tissue culture plate using a micro-pipette. The tissue culture plates were cultured at 37°C in an incubator for 1 day until the cells adhered to the plate wells.

Subsequently, the medium was extracted from the wells and 0.2 ml of the extract was added to each of the following concentrations: 15.1, 31.2, 62.5, 125, 250, 500, and 1000 µg/ml. Three

replicates were prepared for each concentration. Following the protocol outlined by Fresheny (2015). The contents were rinsed with PBS to eliminate any residual dye, and then the cells were allowed to air dry. At a specific length of electromagnetic wave. The wavelength of 492 nm was measured using a spectrophotometer. The subsequent equation has been employed to calculate the rates of growth cells may be suppressed:

Percentage of inhibition rate $= \frac{X-Y}{X} \times 100$ (1)

X= Absorbance of control

Y= Absorbance of each concentration treatment

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

A. Extraction of RNA

In order to extract RNA from cultivated cells, the abm EXCellenCT Lysis Kit (Abm, Canada) was utilized after the cells had been allowed to thaw at room temperature in accordance with the recommendations provided by the manufacturer. Genomic DNA extraction kits, following the manufacturer's instructions provided by TransGen Biotech in China. The Nano-drop spectrophotometer, manufactured by Thermo Scientific in the United States, was utilized in order to ascertain both the purity and the concentration of the cDNA.

B. Primer sequence

The amounts of mRNA for (P53, BCL2, and Caspase-8) and (Hsp90, Hsp70, and Hsp60) in cancer lines Hela and HepG2 and MEF were quantified by qRT-PCR (Agilent MX3005P, Germany), with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serve as a reference gene. All of the genes used in this investigation, are stated in (Table 1).

Table 1: Primer used in gene expression

Human Prime	Forward\ Reverse	5´……...3´ sequence
P53	Forward	CCG TCC CAA GCA ATG GAT G
	Reverse	GAA GAT GAC AGG GGC CAG
Bcl2	Forward	ATC GCC CTG TGG ATG ACT GAG T
	Reverse	GCC AGG AGA AAT CAA ACA GAG GC
Caspase 8	Forward	GAC CAC GAC CTT TGA AGA GCT

	Reverse	CAG CCT CAT CCG GGA TAT ATC
HSP90	Forward	GAT GGC CAA AAA GCA CCT GG
	Reverse	CAG AAG ATA GCA GGG CGG TT
HSP70	Forward	CAA GTG GAC CAG GAG GAA CC
	Reverse	TTC TCG ATT GGC AGG TCC AC
HSP60	Forward	CCC CCC AGA GAC CTC AA
	Reverse	GTC ACT GAG CTG AGA CC
GPADH	Forward	GGG TTC TTT GTG CTG AGC GG
	Reverse	TGC AGA TAG GAA GGG CTT TG

The quantitative real-time PCR (qRT-PCR) targeting the Pin1 polymorphism was conducted using the Rotor-Gene® Q instrument from Qiagen in Germany. Each qRT-PCR reaction involved 2 µl of cDNA, 1 µl for both the forward and reverse primers (with a concentration of 10 µM), and 10 µl of the Perfect Start TM Green qPCR SuperMix kit from TransGen Biotech Co, China. The thermal profile consisted of an initial step at 94 °C for 30 second (one cycle), followed by 40 cycles involving denaturation at 95 °C for 3 second, annealing at 62 °C for GAPDH for 20 seconds, and extension at 72 °C for 10-30 seconds. The final dissociation stage spanned from 55 to 95 °C, with each degree lasting 5 seconds

Novel Cellular Staining with the AO/PI and Fluorescence Microscopy Imaging

The cells were cultivated in a plate with six wells, with a concentration of 1×10^6 cells per well, and they were allowed to adhere for a period of twenty-four hours. There were different distinct concentrations of *C. glomerata* that were applied to the cells for a period of twenty-four hours: 15.1, 31.2, 62.5, 125, 250, 500, and 1000 µg/ml. After the suspension was discarded, the cells were washed twice with phosphate buffered saline (PBS), which is a type of washing solution. Staining was performed using 10 µl of Acridine Orange (AO) for a duration of 15 minutes at room temperature in the dark. Immediately prior to conducting fluorescence microscopic examinations, 10 µl of Propidium Iodide (PI) was added to the cellular pellet. An enhanced Neubauer rhodium hem cytometer was used to analyze cancer cells for apoptotic modification. The cells were examined using fluorescence microscopy, and the IC_{50} that exhibited apoptosis was counted and quantified [17]

Statistical analysis: The data of the current investigation is presented as means. The variations in the inhibitory effects of *C. glomerata* extract on different cell lines were assessed using the F test (ANOVA), and a significance level of $P \leq 0.05$ was applied.

Results and Discussion

Extracting RNA from cancer cell lines and converting it into complementary DNA by reverse transcription

Total RNA was extracted from cells of the HepG2 cancer cell line that were treated and not treated with the crude alkaloid extract at IC50 (300) as well as from Hela cancer cell line that were treated and not treated with the crude alkaloid extract at IC50 level (100 µg/ml) for 24 hours. Also from MEF cancer cell line cells treated and untreated with the crude alkaloid extract at IC50 (1000 µg/ml). All of this RNA was then converted into cDNA. The concentration of cDNA was determined using a Nano-Drop spectrophotometer, and ranged from 1288 to 1312 ng/µL, with a purity of 1.79 to 1.80.

Effect of Crude Alkaliod Extract on Gene Expression of Heat Shock Protein Genes in Cancer Cell Lines

Gene expression level was determined using real-time PCR (qPCR) and Syber green as a fluorescent dye, which can label any DNA, including complementary DNA. Treatment with the crude alkaloid extract at IC50 (300 and 100) µg/mL for 24 h inhibits gene expression of heat shock protein encoding genes in cancer cell lines. Table (3) showed that the gene expression of the Hsp90, Hsp70, and Hsp60 genes encoded in the HepG2 cancer cell line was 0.44, 0.43, and 0.47, respectively. In the Hela cancer cell line, the gene expression levels were 0.11. The values of 0.14 and 0.18 were seen in the treated cells, in comparison to the control group.

Table 3: Change on the Gene Expression in HepG2 and Hela Cancer Cell Lines.

expression Hela	expression HepG2	Gen
0.11	0.44	Hsp90
0.14	0.43	HSp70
0.18	0.47	HSp60

Effect of alkaloid extract on gene expression of programmed cell death genes in cell lines. Treatment with the crude alkaloid extract at a concentration of IC50 for 24 hours led to an increase in gene expression of gene Caspase 8, as the table (4) shows that the percentage of gene expression for gene Caspase8 in cancerous lines reached 1.87, 1.99 and 3.42, respectively, in cells treated with the crude alkaloid extract compared to the control group. While the change in gene expression for the P53and Bcl2 genes in the HePG2 line was 0.17 and 0.51, respectively, and in the Hela cell line, it was 0.22 and 0.64, respectively. While the MEF line was 1.76 and 0.65, respectively.

Table 4: Change in the Gene Expression of Programmed Cell Death Genes P53, Bcl2 and Caspase -8 in Cancer Cell Lines

Expression MEF.	Expression Hela	Expression HepG2	Gene
1.76	0.22	0.17	P53
0.65	0.64	0.51	Bcl2
3.42	1.99	1.87	Caspase- 8

The findings of the present investigation demonstrate that the extract derived from *C. glomerata* Plays a major role in inhibiting the gene expression of heat shock protein genes (Hsp90, HSp70, and HSp60) in HepG2 and Hela cancer cell lines, compared to the control group, this could because. it has anti-cancer and antioxidant activities. *C. glomerata* may prevent oxidative stress-related disorders including metabolic syndrome due to its antioxidant and anti-inflammatory properties [18]. An author indicated that *C. glomerata* extract plays a role in regenerating defective tissues by regulating oxidative stress, apoptosis, and mitochondrial activity [18]. Based on the above investigation, it can be inferred that *C. glomerata* has significant antioxidant and anticancer properties [8]. The methanol extract of the algae, when tested, had remarkable efficacy against HT29 colon cancer cell lines. Additionally, it displayed noteworthy antioxidant capabilities, making it a potential candidate for further investigation as a source of lead compounds. The relationship between oxidative stress and the occurrence of cancer is becoming more supported by scientific evidence in the medical literature [19]. Medical experts often administer antioxidant supplements as part of therapeutic practice. Non-

prescription Antioxidant pills are becoming increasingly popular among the general population. Extensive in vitro and in vivo research has highlighted the importance of naturally occurring antioxidant compounds in preventing the formation of free radical compounds. This, in turn, helps to prevent DNA damage and the formation of mutant genes and proteins, significantly reducing the risk of developing cancer [20]

Several chemicals derived from marine algae have been scientifically demonstrated to exhibit significant anti-cancer properties against different types of cancer cells. In their study, Han *et al*. (2022)[21] demonstrated the efficacy of extracts derived from the marine alga *Thalassia testudinum* in inhibiting the growth and advancement of colon cancer in live organisms. The processes responsible for this action were determined to be the suppression of blood vessel formation, activation of cellular self-degradation, and enhancement of the body's natural defence against cancer. In a recent study, Zhang *et al*. (2021)[22] demonstrated the anticancer and antimetastatic effects of the marine chemical penisuloxazin in an in vitro breast cancer model.

Many researchers noted that various preparations of the marine algae *C. glomerata* have remarkable antioxidant superoxide activity. The Chloroform extract of the sea algae exhibits the highest SOD dismutase activity among the extracts, with a value of 98.25±0.93 U/mg protein. Researchers hypothesised that marine algae have developed robust antioxidant defence systems to counteract the rising pollution and contamination in the marine environment [8]. Heat shock proteins (HSPs) perform various functions during times of stress, including their ability to be released into the extracellular environment. Once there, they survey the surrounding area, control the activity of extracellular proteins, and transmit autocrine and paracrine signals. Cancer cells undergo chronic stress due to their disrupted homeostasis and behaviour, leading to continuous secretion of heat shock proteins. In addition to promoting anti-tumour immunity, extracellular heat shock proteins (eHSPs) can also contribute to the proliferation and aggressiveness of cancer cells by sustaining many cancer hallmarks. eHSPs play a role in modifying the extracellular matrix, preventing cell death, facilitating cell movement and invasion, triggering a change in cell phenotype from epithelial to mesenchymal, promoting the

growth of new blood vessels, and activating supportive cells in the surrounding tissue, all of which ultimately contribute to the spread of metastatic cancer cells. Increased comprehension of eHSP (extracellular heat shock proteins) activity and their role in tumour growth and progression is creating novel possibilities in cancer diagnostics and treatment [23].

Current study is being conducted to find novel pharmaceuticals for the treatment of human inflammatory disorders (HIDs) and cancer, with a specific emphasis on identifying medications that will have less adverse effects. Heat shock proteins (HSPs) have recently garnered significant scientific attention due to their ubiquitous presence in several human disorders, including individuals who have undergone HID testing, despite the fact that their role in certain HIDs remains uncertain. Authors propose that focusing on HSPs in Human Infectious Diseases (HIDs) could be promising candidates for the treatment and control of various HIDs, as well as for the early identification of these diseases [19].

Results of present study showed the *C. glomerata* extract plays a major roles in inhibition of gene expression of programmed cell death genes (*P53 and Bcl2*) in cancer cell lines HepG2 and Hela, but increase gene expression (MEF) cell line compared to control gene expression (normal value of control gene expression is 1.00). In contrast, we showed the *C. glomerata* extract is leading to increase *Caspase 8* gene in cancer cell lines HepG2 , Hela and MEF cell line compared to control gene expression (Table 2), due to *C. glomerata* has regulatory effect.

Researchers discovered that silver nanoparticles (AgNPs) can be produced using the biogenic synthesis method utilizing marine algae *C. glomerata* extract. These nanoparticles were found to have a specific apoptotic effect on colon cancer HCT-116 cells, indicating their potential for targeting therapy in colon cancer treatment [24].

Targeting the apoptotic pathway is a compelling strategy for discovering novel anticancer treatments, as it is not limited to a single type of cancer. Cancer is characterized by the presence of many abnormalities in both the extrinsic and intrinsic pathways, enabling cancer cells to avoid apoptosis, a key feature of the disease. Enabling the precise targeting and activation of an apoptotic pathway would offer a broader and more universally applicable approach to cancer

treatment. Several plant-derived chemicals that are non-toxic to healthy cells show great potential in inducing apoptosis [11].

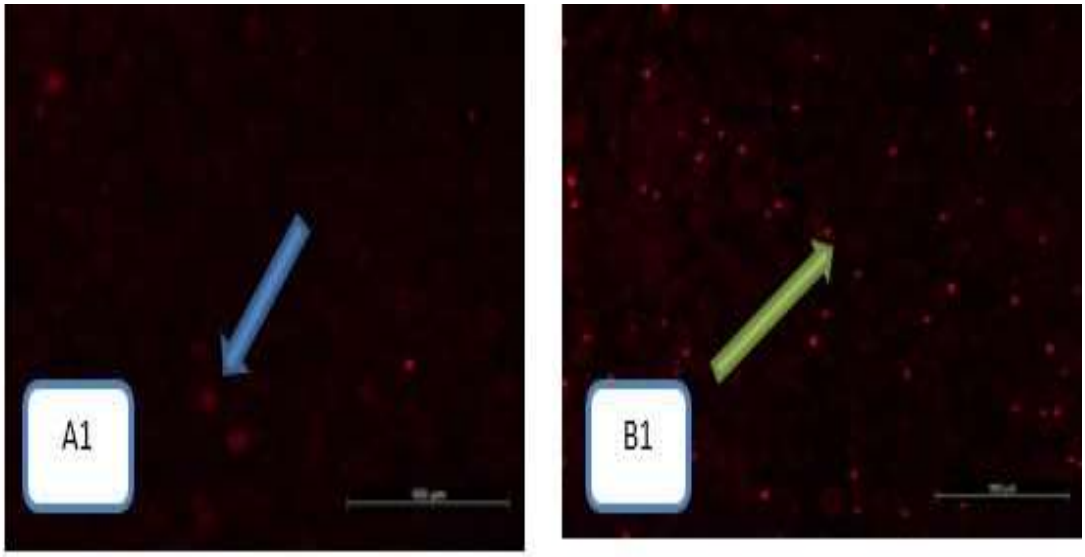
Detection the morphological alterationof the cells

The effects of the crude alkaloid derived from *C. glomerata* on HepG2, Hela, and MEF cell lines was seen through inducing cell death during examination using fluorescence microscopy, as shown in Figures (1, 2, and 3), using the doublet (AO\PI). This was observed when the cells were exposed to the extract for a period of twenty-four hours at an IC50 concentration. The use of fluorescence microscopy, which can distinguish between normal cells and cells that have perished as a result of programmed cell death, or direct observation are both methods that can be utilized. Both the evaluation of morphological alterations (such as nuclear modifications and apoptotic bodies) and the differentiation between live cells and dead cells that have experienced apoptosis have been demonstrated to be possible through the utilization of this technology. All of the nuclei of living cells in the control group, which consisted of cells that had not been treated, appeared green in colour, with a typical spherical shape and regular chromatin. This was because the AO dye was able to penetrate both the cell membrane and the DNA bands inside the cells. On the other hand, the nuclei of cells that had undergone apoptosis were red, with broken nuclei and dense chromatin. Apoptosis occurs through many biological processes. The cell kills itself in a programmed manner according to a series of cellular processes. This process leads to the elimination of abnormal cells[25]. It differs from the other type of cell death called necrosis, which leads to swelling, enlargement, and rupture of dying cells, DNA fragmentation, protein cleavage, and protein cross-linking are biochemical features of apoptosis, DNA fragmentation is a predominantly characteristic feature of programmed cell death [26].

A study conducted by Permatasari *et al.* (2022) [27] showed the toxicity of the methanolic extract of *Caulerpa racemosa* algae, which is considered one of the basic algae that contains biologically active metabolites such as alkaloids, terpenes, flavones, and tannins. It was found to have an anti-growth effect on HeLa cancer, as well as to have an activity that supports

programmed cell death, as it was shown that the metabolites It affects microtubule dynamics, metabolic pathways in organelles within the cell, mitochondrial health, protein phosphorylation, and DNA synthesis. There is another study conducted by Bourebaba *et al.* (2019)[18] showing that the methanolic extract prepared from the biomass of *C. glomerata* algae in different concentrations acts as an antioxidant for mesenchymal stem cells derived from adipose cells in horses, as it works to reduce oxidative stress resulting from regulating the state of programmed cell death and regulating the Reduction of the expression of pro-apoptotic genes (P53, P21, Bax and Caspase9) and significantly reduced mitochondrial dysfunction.

These studies show the effect of algae extracts on inducing the process of programmed cell death in cancer cells through their effect on cellular indicators.



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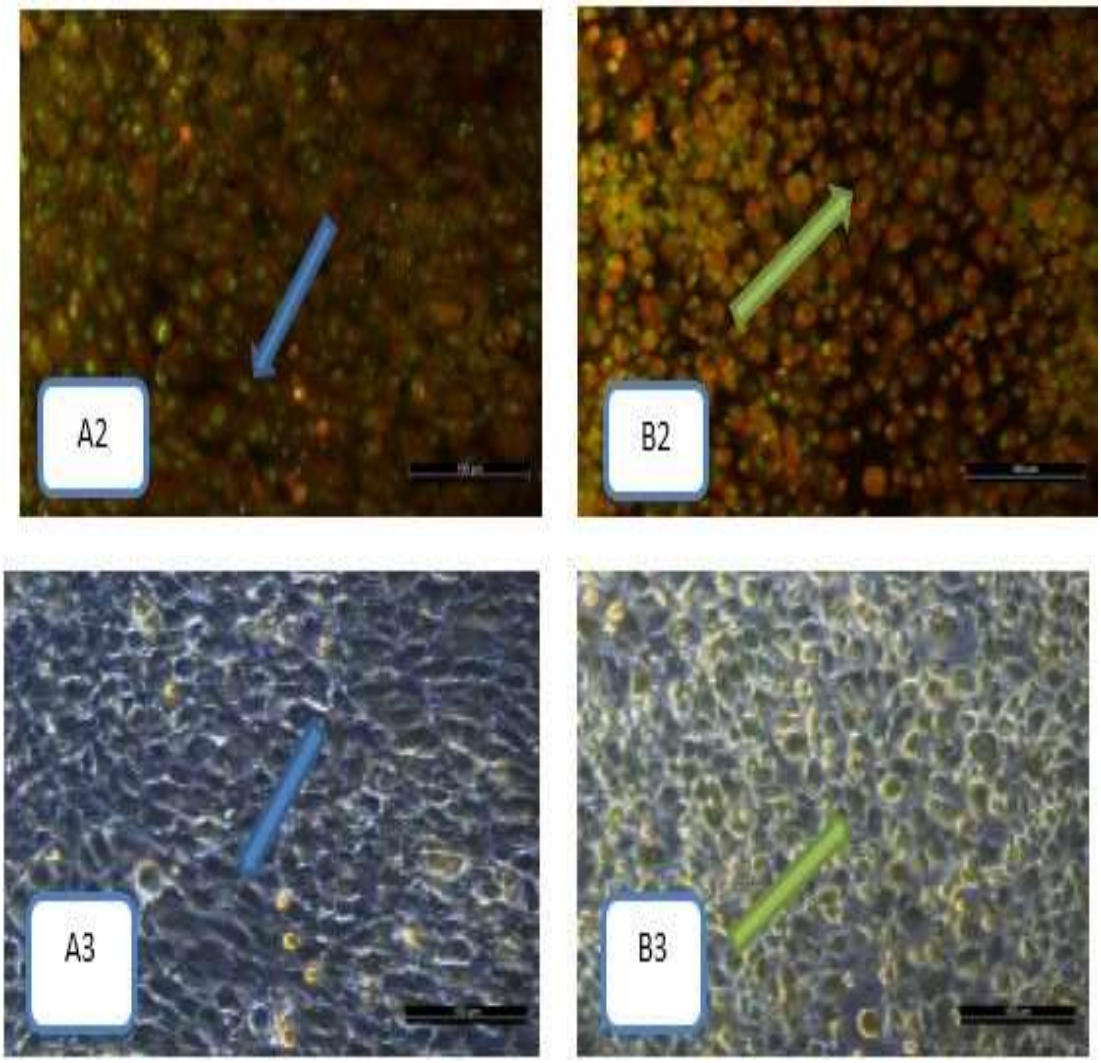

Figure 1: Photomicrographs using inverted fluorescent microscopy using (AO/PI) dye in HepG2 cells treated with IC50 of crude alkaloid extract. A: represents cells untreated with the crude alkaloid extract (control group); B): Represents cells treated by utilized the crude alkaloid extract.

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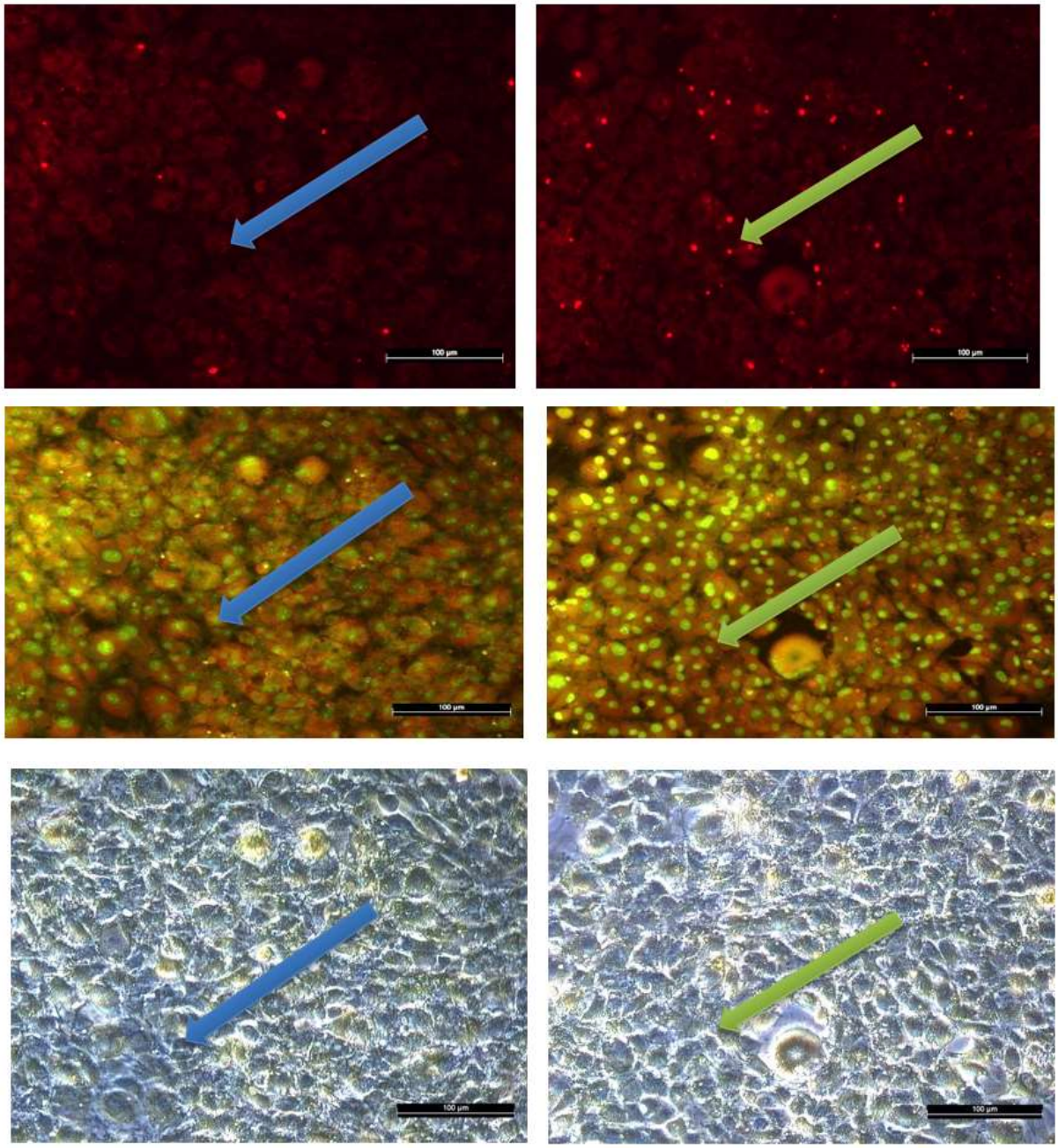

Figure 2: Photomicrographs using inverted fluorescent microscopy using (AO/PI) dye in Hela cells treated with IC50 of crude alkaloid extract. A: represents cells untreated with the crude alkaloid extract (control group); B): Represents cells treated by utilized the crude alkaloid extract

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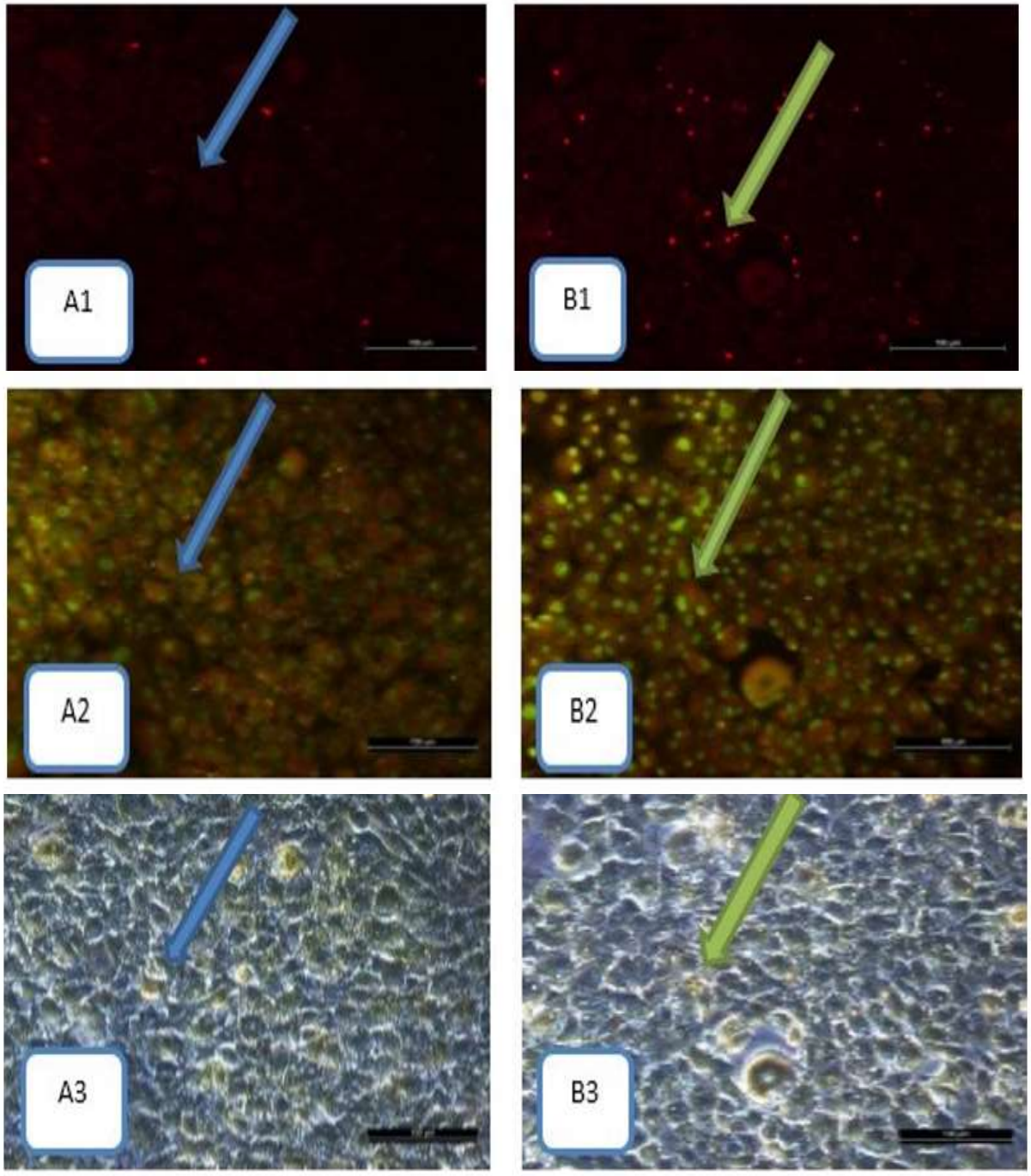

Figure 3: Photomicrographs using inverted fluorescent microscopy using (AO/PI) dye in MEF cells treated with IC50 of crude alkaloid extract. A: represents cells not treated with the crude alkaloid extract (control group); B): Represents cells treated by utilized the crude alkaloid extract.

Conclusions

The *Cladophora glomerata* extract may playing a major role in treating cancers (HepG2 and Hela cell lines) through regulation of heat shock proteins genes (*Hsp90*, *HSp70*, and *HSp60*) and programmed cell death genes (*P53* ,*Bcl2*, and *Caspase 8*) that have grate roles in cancer.

Recommendations

Studying the activity of *Cladophora glomerata* extract in inhibition gene expression of *BRCA1*, *CHEK2*, and *PALB2* gens in Breast cancer in men, and *MLH1*, *PMS2* ,and *PTEN* genes in Endometrial cancer.

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Ethical clearance: The samples were gained according to Local Research Ethics Committee approval in the College of Science, University of Diyala, No. 22EC02 in 61/8/2022.

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