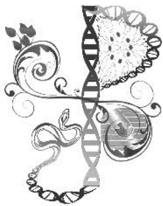

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Original Article

Evaluation of Hydroalcoholic Extract of *Avicennia marina* Effect on Viability and Expression of Genes Involved in the Apoptotic Pathway of Glioblastoma Cancer Cell Line

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Abstract

Avicennia marina is known as the main species of mangrove forests in Iran. The study of biological processes shows that the presence of a wide range of phytochemical compounds in this plant has made it an excellent candidate for use in therapeutic applications. The purpose of this study was to investigate the cytotoxicity and apoptosis induction of hydroalcoholic extract of *A. marina* on human glioblastoma and normal fibroblast cell lines. With this aim, the hydroalcoholic extract of *A. marina* leaves were extracted firstly and then the target cells were treated with different concentrations of the extract. Cell viability percentage of treated normal and cancer cells was assessed using MTT assay at 24, 48 and 72 h. Also, the half maximal inhibitory concentration (IC_{50}) values of hydroalcoholic extract were calculated at the times of testing for both cancer and normal cell lines. Quantitative PCR (qPCR) was used to evaluate the expression of *Bcl2* and *Bax* apoptotic genes and dual staining (AO / EB) was used to stain the nucleus of target cells for imaging by a fluorescence microscope. The results of cytotoxicity tests showed that the hydroalcoholic extract of *A. marina* is able to inhibit the metabolic growth of more than half of U-87 MG cells at a concentration of 0.57 mg/ml. It was also found that hydroalcoholic extract has a higher effectiveness in inhibition of cancer cells proliferation compared to normal cells. Analysis of *Bcl-2* and *Bax* genes expression levels in cells treated with *A. marina* extract showed a significant decrease in *Bcl-2* anti-apoptotic gene expression levels ($P < 0.01$) and an increase in *Bax* apoptotic gene expression levels ($P < 0.001$). Eventually, the results of the dual staining study of the cell nucleus clearly depicted the morphological changes in different stages of apoptosis and confirmed the results of the qPCR and MTT assay.

Keywords: *Avicennia marina*, Glioblastoma, Cytotoxicity, Apoptosis, MTT Assay, Cancer.

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1. Introduction

In recent era cancer has been known as one of the most challenging diseases and one of the leading causes of death worldwide. It is estimated that by 2025, the number of cancer patients will be enhanced to more than 20 million per year [1]. Currently, chemotherapy

and radiation therapy are the most common methods for treating cancer. In recent years, bioactive compounds extracted from medicinal plants have drawn attentions of many pharmaceutical industries. Moreover, there are lots of findings that have shown the gradual decline in the effectiveness of synthetic drugs against several pathogens and this issue has led to decreased willingness of patients to use synthetic drugs due to their destructive side effects; as a result, scientists have increasingly focused on the production of drugs based on natural bioactive substances [2, 3]. Nowadays, more than 70% of the produced anticancer drugs have herbal origin, such as Paclitaxel and Vinblastine [4]. *Avicennia marina* (Forssk.) Vierh (*A. marina*) of the genus Avicennia L. and the family Acanthaceae, is a tree/shrub with green branches and leaves. In this plant, the leaves are simple, opposite and thick, lanceolate and oval in shape with a glossy upper surface and a white underside with many hairs. In addition, the leaves and branches of this species are dense and scattered. The flowers are small, yellow and fragrant (fig. 1).

Figure 1. Image taken from a branch of the *A. marina* located on Qeshm Island, Iran. the leaves are simple, dense, sprayed, opposite and thick in the shape of a spear and oval with a glossy surface and yellow flowers in small size can be seen.

A. marina along with *Rhizophora mucronata* as the main and exclusive species have been distributed in the mangrove forests of Iran from Goiter Gulf to Nayband Gulf [5]. This plant is adapted to specific ecotone conditions and waterfront regions, which is very important in the study of biological processes. Comparing with terrestrial plants these herbs are able to absorb CO_2 more than 5 times and consume it through intracellular biochemical processes such as photosynthesis [6, 7]. *A. marina* is highly resistant to severe environmental stresses and able to tolerate very high levels of temperature and salinity (up to twice the sea salinity) [8-10]. The survival and such unique morphological, biological, ecological and physiological adaptation in stressful and variable environmental conditions are due to the presence of a wide range of phytochemical compounds with valuable biological properties in these plants. Benefiting of these compounds has made the mangrove species an excellent candidate to be used in therapeutic applications. Since the distant past, people in the southern regions of Iran and other countries have emerged with the potential characteristics of different parts of the mangrove plants to treat various diseases. For example, leaves have been used in treatment of diseases such as arthritis pain, abscesses, rheumatism, snake bites and wounds; decoction of aerial parts and leaves for fighting against malaria and food poisoning; aqueous extract for treating sore throats; stem bark, root paste and fruit for treating digestive disorders, skin ulcers, scabies and some fungal infections; as well as wax as an antifungal drug for relieving toothache [11-13]. As the presence of

triterpenoids was first reported from mangroves [14], more than sixty compounds have been extracted from these plants. So far, the presence of several bioactive compounds have been confirmed such as terpenoids and steroids, naphthalene derivatives, flavonoids, carbohydrates, triterpenes, iridoid glycosides, phenylpropanoid glycosides and flavonoids [15]. Besides, the antioxidant, antiviral, antifungal and antibacterial effects of this plant have also been approved [16]. However, a few researches have been performed to determine the mechanism of inhibition of cancer cells by different species of mangrove plant. In particular, the focus on the study of the molecular mechanisms of cell death is very limited. In this regard, currently the anti-cancer properties of *A. marina* and *Avicennia germinans* on breast and laryngeal cancers have been studied to a limited extent [17]. In a study, bioactive molecules in the fruit of *A. marina* were studied, which led to the identification of 29 known compounds and a new triterpenoid saponin. Pharmacological experiments showed that the new triterpenoid saponin had cytotoxic properties on human glioma stem cells. furthermore, a wide range of isolated compounds have potential of antioxidant activity [18].

In this study, the cytotoxicity of leaves ethanolic extract of *A. marina* on human glioblastoma cancer cell line and human normal fibroblast cell line was investigated using the tetrazolium bromide (MTT) method. In the next step, the induction of apoptosis in these cancer cells was evaluated by examining the expression of genes involved in the apoptotic pathway (*Bcl2* and *Bax*).

2. Materials and Methods

2.1. Chemicals and Reagents

DMEM (Dulbecco's modified Eagle's medium), fetal bovine serum (FBS) and Phosphate Buffered Saline (PBS) procured from Thermo-fisher scientific Co. (*Waltham, MA,* USA), trypsin-EDTA enzyme (0.25%) and penicillin G/streptomycin, DMSO (cell culture grade), dimethyl thiazolyl tetrazolium bromide (MTT) procured from Sigma-Aldrich Co. (St Louis, MO, USA). Acridine orange, Ethidium bromide procured from Thermo-fisher scientific Co. (*Waltham/Boston, MA,* USA). Ethanol and other solvents and reagents (such as total RNA isolation and Real Time PCR Master Mix) procured from Sinacolon, Co., Iran.

2.2. Preparation of Leaf Extract

Leaves of *A. marina* were collected from the Mangrove forests of Persian Gulf area that is located on the southern coast of Iran (Qeshm Island, 26°46'26.0" N 55°43'35.6" E). The collected samples were identified in terms of botanical classification and kept as a herbarium sample at Biotechnology Center, University of Hormozgan, Iran (Document No.: 34218). Collected healthy leaves were washed with sea water and sterilized with distilled water to remove epiphytes and other external impurities. The leaves were air-dried in shade at room temperature and powdered using powder mills. 10 g of mixed leaf powder were used to obtain extract with 100 ml of 95% ethanol with stirring for 48 h. The extracts were filtered with

Whatman No. 1 filter paper and centrifuged at 8000 rpm for 15 min. Vacuum rotary at 45 °C was used to concentrate the samples. Then, the concentrated samples were dried with a freeze dryer at -70 °C for 72 h and Finally, the dried samples were dissolved in dimethyl sulfoxide solution (DMSO) and stored in darkness at -20°C.

2.3. Cell Culture

U87MG (human glioblastoma cell line as cancer group) and HFFF2 (normal human fibroblast cell line ; as a normal group) cell lines were purchased from the Iran Cell Bank, Pasteur Institute. Cells were maintained in DMEM supplemented containing 10% FBS and 1% penicillin G and streptomycin antibiotics in a 5% CO_2 humidified atmosphere at 37 °C [19].

2.4. In-vitro Cytotoxicity Assay

The *in vitro* cytotoxic activity of hydroalcoholic extract was evaluated using 3-(4, 5-dimethyl-thiazol-2 yl)-2, 5- diphenyl tetrazolium bromide (MTT) assay [20]. In brief, the monolayer containing about 6×10^3 cells were added to a 96-well microplate wells and incubated overnight at 37 °C in 5 % CO_2 athomospher. Cancer and normal cells were treated with hydroalcoholic extract, at concentrations of 30.25, 62.5, 125, 250, 500 and 1000 µg/ml and incubated for 24, 48 and 72 h (Primary hydroalcoholic extract was obtained by dissolving 1 mg of dry weight of the leaf extract in 1 ml of DMEM medium containing 0.1% DMSO, 10% FBS, and 1% penicillin G and streptomycin antibiotics). Moreover, untreated cells and 0.1% DMSO solvent were used as a positive and negative controls, respectively. At the end of the incubation time, the medium was discarded and about 25 µl of MTT (5 mg/ml) solution were added to each well and further incubated for 4 h in the CO_2 incubator. After that, the MTT solution was aspirated, 150 µl of DMSO were added to each well to dissolve the formazan crystals and the optical density of the solution was measured at 570 nm using ELISA microplate reader (Model ELx808, BioTek, USA). For the cells viability calculation, readings were performed from an average of three replicates for each sample then, the culture medium background subtracted from assay readings (This is the corrected absorbance). Eventually, the percentage of cytotoxicity was calculated by the following equation:

$$\% \text{ Cytoxicity} = (100 \times (\text{control - sample})$$

2.5. Quantitative Evaluation of Genes Expression

Quantitative PCR analaysis was used to evaluate the expression of genes involved in the apoptotic pathway. To do so, about 5×10^4 U-87 MG cells were incubated in each of the 12-well cell culture plates for 4 h to adhere to the bottom of the plate. After that, the medium was aspirated and replaced with fresh medium containing 0.57 mg/ml of hydroalcoholic extract and incubated in CO_2 for 48 h (during this period, the morphology of the cells was observed and evaluated using a reverse microscope at specified intervals). At the end of the test time, the medium containing hydroalcoholic extract was discarded and the

cells' surfaces were washed with 1 ml PBS. Total RNA was extracted from the samples using RNX-plus reagent (Sinacolon Co.), according to the manufacturer's instructions. Then, RNA pellet was dissolved in 5 µl RNA storage solution and stored at −80°C until use. To determine the concentration and quantify the amount of extracted RNA, the method of light absorption of solution containing RNA at 260 and 280 nm was used. In the next step, the extracted RNA was reversed to transcription of cDNA using the kit EasyTM cDNA synthesis (Pars tous Co) (according to the manufacturer's instructions). Gene specific primers were designed by Oligo 7 software and for the amplification of the mRNA transcripts of the *Bax* and *Bcl2* (target genes), as well as for *GAPDH*, which was used as a reference gene (Table 1). In this study, a cybergreen probe was used to observe the real-time PCR process.

2.6. Assessing the Occurrence of Apoptosis

In this study, dual staining with fluorescence dyes including ethidium bromide and acridine orange (AO / EB) were applied to stain the nucleus of target cells. A healthy cell membrane does not allow EB to penetrate into the cell, but AO can penetrate the cell membrane and thus enter the cell nucleus. AO emits fluorescent light by binding to DNA. Apoptosis has two stages. In the early stages, all living cells are usually permeable to AO and the nuclei of cells are visible in orange fluorescence (inclusive staining). At this stage, the membrane still has its selective permeability and does not allow EB to enter the cell (exclusive staining) [21]. However, AO was still able to penetrate the cell membrane barrier and show DNA fragmentation of the apoptotic cell. In the late stage of apoptosis that has been called secondary necrotic stage as well, the cell membrane loses its selective permeability, and the EB was thus able to penetrate into the nucleus, and bind to DNA to emit red fluorescence. Cancer cells were added to a final concentration of 3×10^4/ml, 96-well microplate wells and incubated overnight at 37 °C in 5 % CO_2 atmosphere, the rests have remained untreated or treated with 0.57 mg/ml of hydroalcoholic extract.

Table 1. Specimens of target (*Bax* and *Bcl-2*) and reference (*GAPDH*) primers.

Gene name	Sequence	Primer	Primer length (bp)	Sequence length (bp)
Bax	5' AAGAAGCTGAGCGAGTGTCT3'	F	20	236
	5' GTTCTGATCAGTTCCGGCAC3'	R	20	
Bcl-2	5'CTG AGT ACC TGA ACC GGC A3'	F	19	106
	5'GAG AAA TCA AAC AGA GGC CG3'	R	20	
GAPDH	5'AAG GTG AAG GTC GGA GTC AA3'	F	20	108
	5'AAT GAA GGG GTC ATT GAT GG3'	R	20	

The samples in a 96-well plate were divided into 2 groups, with 12 well samples in each group corresponding to control and treated groups. After being cultured for 48 h, 20 µl of trypsin were added into each well. When cells had sloughed off, their suspensions (15 µl) were transferred to glass slides. Dual fluorescent staining solution (5 µl) containing 100 µg/ml AO and 100 µg/ml EB (AO/EB, Sigma, St. Louis, MO) were added to each suspension and then covered with a coverslip. The morphology of apoptotic cells was examined where 200 cells were counted within 10 minutes using a fluorescent microscope (Olympus CKX53, Japan). The dual AO/EB staining method was done in triplicates. The percentage of live cells was calculated using the following relation:

Percentage of live cells = total number of live cells / total number of live and dead cells ×100

2.7. Statistical Analysis

Statistical tests of this research were performed using version 22 of SPSS software. Data were presented as the mean standard deviation of independent experiments. One-way ANOVA was used for statistical analysis of data and the value of $p<0.05$ was considered as a significant level in all tests. To measure IC_{50}, a linear graph with regression coefficient above 0.9 was prepared. Also, GraphPad Prism 8 software was used to fit a dose response curve to determine the IC_{50}. All studies were performed with three replications in the form of control, normal and cancer cell line groups treated with different concentrations of hydroalcoholic extract.

3. Results and Discussion

Viability percentages of normal and cancer cells treated with hydroalcoholic extract of *A. marina* were assessed using MTT test at 24, 48 and 72 h. In fig. 2, the results of descriptive statistics show a significant reduction in the survival rate of cells treated with hydroalcoholic extract of mangrove compared to the control group (without treatment + 0.1% dimethyl sulfoxide). The effect of hydroalcoholic extract on inhibiting the growth of cancer cells is significant.

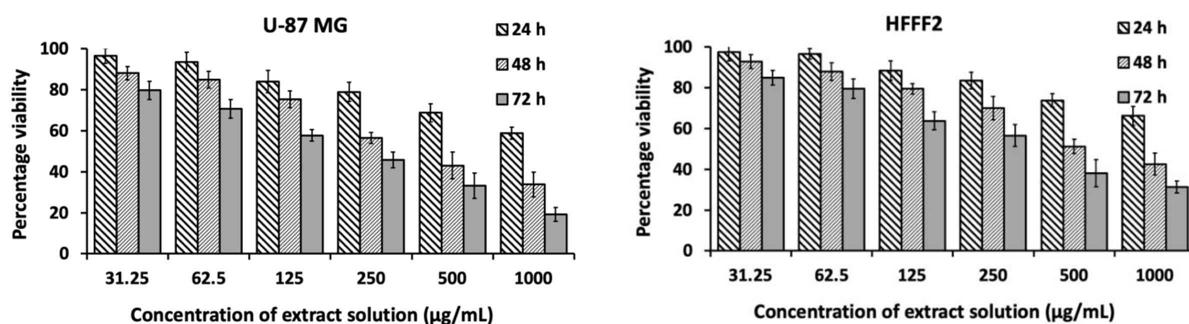

Figure 2. Comparison of viability rates of U-87MG and HFFF2 cells (left to right) in the presence of different concentrations of hydroalcoholic extract at 24, 48 and 72 h. The error bar was calculated based on mean ± SD, (n = 4).

Inhibition of 50% and 75% of the cell population were observed at 0.57 and 1.02 mg/ml concentrations of hydroalcoholic extract, respectively, after 48 h culture ($p < 0.05$). The results of cytotoxicity indicate that the reduction in the viability has been affected by two factors of treatment concentration and time. Furthermore, the results show that in equal concentrations, the cytotoxicity of hydroalcoholic extract on cancer cell line (U-87 MG) is greater than on normal cell line (HFFF2). This can be related to various biological factors at the cellular and molecular levels such as: cell type and position, more sensitivity and specificity to tumor cell surface receptors, differences in morphological characteristics, differences in permeability between normal and cancer cell membranes, rate of metabolic activity of tumor cells, or other biological factors [22]. The study of the occurrence of any of the mentioned factors can explain part of the molecular mechanism or cellular signaling pathways involved in the cell, as suggested previously [23]. Moreover, the results of cytotoxicity studies and half maximal inhibitory concentration (IC_{50}) (Table 2) show that the hydroalcoholic extract has lead to toxicity on glioblastoma cancer cells, butproduced only partial toxicity on normal cells.

Table 2. IC_{50} values of hydroalcoholic extract at different times in normal and cancer cell lines (n = 4).

IC_{50} (mg/mL)		
HFFF2	**U-87MG**	**Time (h)**
1.40	1.14	24
0.73	.057	48
0.50	0.35	72

One solution to overcome this challenge as indicated by previous researchers seems to be the use of intelligent drug carriers and smart stimuli-responsive drug delivery systems which are sensitive to various stimulus such as pH, light, temperature, magnet, redox etc [24]. In addition, the use of nanotechnology for delivering bioactive compounds, which have the ability to specifically target cancerous tissues, and releasing the drug cargo to the desired sites, can also be considered as a suitable solution. In this way, normal cells will be less exposed to the drug and as a result will show fewer side effects.

In Fig. 3, the results of morphological changes of U-87MG and HFFF2 cell lines after treatment with hydroalcoholic extract can be seen (a reverse microscope was used for imaging; Olympus CKX53, Japan). Images of U-87MG cell line treated with hydroalcoholic extract shows deformation and shrinkage of the cell membrane and size reduction in the cytoplasm. Also, the density of cells adhering to the culture medium in cancer cells treated with hydroalcoholic extract was reduced compared to the control group. In contrast, the group of HFFF2 cell line, which was treated with hydroalcoholic extract, morphological changes in the surface of the membrane and deformation were less than changes in the control group, and the cells were natural and spindle-shaped. However, the surface density of HFFF2 cell line adhering to the culture medium, such as observed in the U-87MG cell line, has been decreased in comparison with their control group.

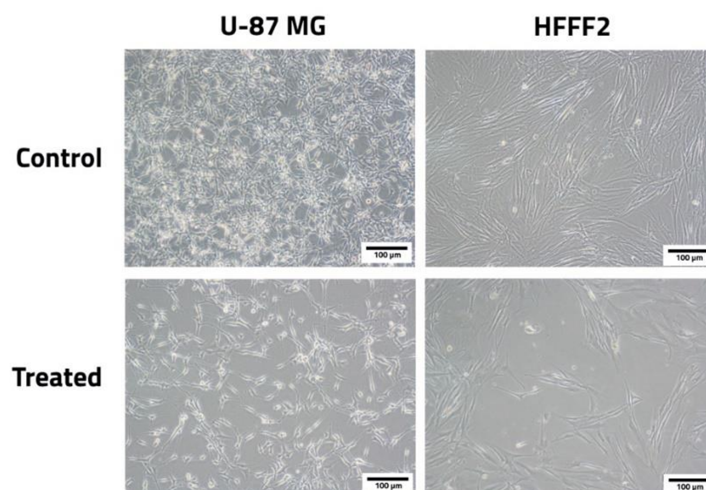

Figure 3. Inverted microscope images of comparison of morphological changes of U-87MG and HFFF2 cell lines treated with hydroalcoholic extract (IC50 = 0.57 mg/ml) with control group after 48 h (x 10 magnification).

The results of comparing the relative expression of target genes (*Bax* and *Bcl-2*) between control and treatment groups with effective dose of extract (at IC_{50} = 0.57 μg/ml) were considered, relative to the expression of *GAPDH* reference gene in U-87 MG cell line after 48 h, and the results can be seen in Fig. 4.

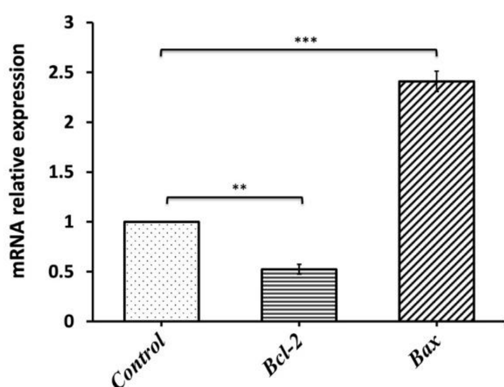

Figure 4. Effect of hydroalcoholic extract (IC50 = 0.57 mg/ml) on expression of *Bax* and *Bcl-2* genes against U-87 MG cell line after 48 h. Cells containing 0.1% dimethyl sulfoxide were used as a control group. ** ($p < 0.01$) and *** ($p < 0.001$) are considered as significant criteria of treatments in comparison with the control group.

The results clearly show a significant decrease in *Bcl-2* gene expression ($p < 0.01$) and a significant increase in *Bax* gene ($p < 0.001$). In this study, *Bcl-2* and *Bax* genes were thus studied as the candidate genes of the apoptotic pathway. *Bcl-2* gene is located on chromosome 21q18 and its protein regulates the activity of caspase enzymes [25]. In the absence of apoptotic signals, the Bcl-2 protein interacts with the Bax pro-apoptotic protein in the mitochondrial membrane and forming a heterodimer, thus prevents the induction of apoptosis. However, in the presence of apoptotic signals, the Bcl-2 protein binds to the Bad proapoptotic protein and prevents it from interacting with the Bax proapoptotic protein. As a result, Bax protein is able to form hemodimers in the mitochondrial membrane and create pores in that membrane, destroys the membrane potential and releases cytochromes C and apoptosis-inducing proteins (Apoptotic protease activating factor) [26]. Finally, the apoptosome complex is known to be formed by

the binding of cytochromes C, apoptotic protease activating factor and procaspase 9, followed by the activation of caspase 3, 6 and 7 cascades [27, 28]. Analysis of *Bcl-2* and *Bax* genes expression in cells that were treated with hydroalcoholic extract show a significant decrease in *Bcl-2* gene expression and an enhancement in *Bax* gene expression. And since *Bcl-2* and *Bax* are known as anti-apoptotic and apoptotic genes, respectively, the changes in their expression can be attributed to the apoptosis phenomenon [29]. Previous studies have shown that *A. marina* leaf extract was also cytotoxic to breast cancer cells via apoptosis [2]. In a study, potential targeting of Hep3B liver cancer cells by lupeol isolated from *A. marina* was investigated. The efficacy of lupeol was investigated by measuring its impact on key players in cancer advancement and movement, Bcl-2 anti-apoptotic and Bax pro-apoptotic proteins. Lupeol downregulated *Bcl-2* expression, contributing to the induction of apoptosis, though it caused no impact on *Bax* expression in Hep3B cells [30].

AO/EB dual staining method was used to assess the occurrence of apoptosis. Imaging was performed with a fluorescence microscope at x 40 magnification. The images in Fig. 5 clearly shows the early and late stages of apoptosis and necrosis in U-87 MG cell line treated with hydroalcoholic extract (IC_{50} = 0.57 mg/ml) after 48 h. In Fig. 5 (a) nucleus of the control group cells (untreated) are shown in green (healthy nuclei) and Fig. 5 (b) the green nuclei with thick chromatin and the yellow-orange nuclei shows the early stages of apoptosis and where the orange nuclei tend to red indicate the late stages of apoptosis and red nuclei represent necrotic cells.

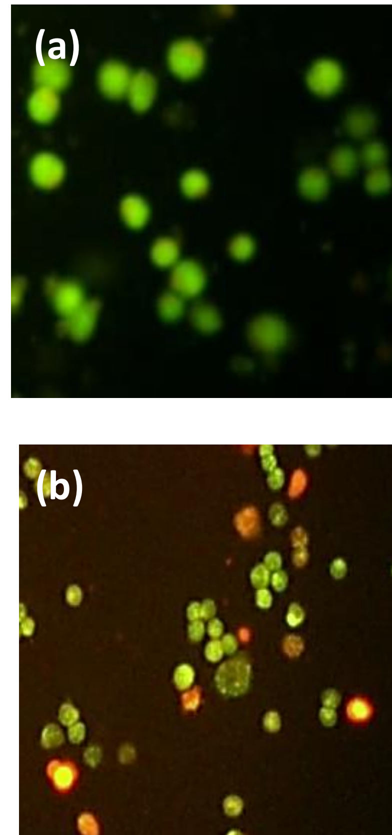

Figure 5. Fluorescence microscope images to qualitatively determine the occurrence of apoptosis. (a) control group (untreated cells) compared to (b) U-87 MG cell line treated with hydroalcoholic extract (IC_{50} = 0.57 mg/ml) after 48 h. Scale bar = 40 μm.

4. Conclusion

The results of this study show that the hydroalcoholic extract of *A. marina* leaves are effective in inducing apoptosis and inhibiting the proliferation of human glioblastoma cancer cells. It was also found that reduction of cancer cell population was directly related to the concentration and treatment time factors. On the other hand, these effects indicate that hydroalcoholic extract of *A. marina* leaves could be used as a suitable candidate for further

studies on other cancer cell lines as well as *in vivo* studies too. In addition, as a suggestion for the continuation of this study, the effectiveness and cytotoxicity of the obtained hydroalcoholic extract loaded in several target delivery systems can also be studied and its results can be compared with some free drugs for further analysis.

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