Phytochemical Analysis, Computational Modeling and Experimental Evaluations of Avicennia Marina Anti-cancer Activity on Breast, Ovarian and Cervical Cancer Cell Lines Running Title: Anti-cancer Activity of Avicennia Marina

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Research Article

Keywords: Avicennia marina, anti-cancer, mangrove, apoptosis, caspase, cell cycle arrest

Posted Date: September 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-835233/v1

Abstract

Background: *Avicennia marina*, the gray mangrove, is an herbal source of bioactive anti-cancer compounds.

Purpose: The present study aimed to evaluate phytochemical compositions of ethanol and ethyl acetate extracts of *A. marina* leaves and experimental study and computational modeling of their anti-cancer activity on breast, ovarian and cervical cancer cell lines.

Study design: Phytochemical analysis, computational modeling and experimental in vitro evaluation

Methods: Phytochemical analysis and GC-MS analysis were used to detect phenolic and flavonoid contents in ethanol and ethyl acetate extracts of *A. marina* leaves. Cell proliferation, viability, cycle and western blot assays of the extracts were performed on MCF-7, OVCAR3, and HeLa. Computational modeling done to detect effective compounds.

Results: The extracts of *A. marina* leaves had high phenolic and flavonoid contents. In GC-MS analysis of the extracts, anti-cancer and anti-proliferative compounds were detected. Moreover, after treatment of MCF-7, OVCAR3, and HeLa, separately, the MTT assay, cell proliferation assay, and cell viability assays showed anti-proliferative activity, decreasing of cell population and decreasing cell viability, respectively. In addition, the cell cycle analysis showed that the S phase of the cell cycle increased in MCF-7. Moreover, the western blot analysis showed that the pro-apoptotic cell effectors such as Bax and caspase-1, -3, and -7 increased. Computational results of affinity of ligands detected by GC-MS compounds and stimulated apoptosis effectors detected by western blot showed five molecules in *A. marina* leaves playing role in OVCAR3 and HeLa apoptosis.

Conclusion: The extracts of *A. marina* leaves have anti-cancer effects on breast, ovarian and cervical cancer cells via cell cycle arrest or apoptotic mechanisms.

Introduction

Breast cancer, cervical cancer, and ovarian cancer are the first, the second, and the fourth most common female-specific cancers in worldwide, respectively [1–3]. An average of 51% of various types of cancer patients uses complementary and alternative medicine [4]. The herbal medicine, as a common approach to cancer treatment, showed various species of herbs have anti-cancer, anti-metastatic, anti-proliferative, anti-oxidant and/or pro-apoptotic effects [5]. Mangrove forests are known as one of the most productive ecosystems in the world, and they grow mainly in the tropical or sub-tropical regions [6]. Mangrove plants have been known as resources of traditional and herbal medicine [7]. Mangrove includes several kinds of phytochemical compounds which are being used for various diseases treatments [8].There are rich sources of the seven most common chemical constituents in mangrove species, including terpenoids, tannins, steroids, alkaloids, flavonoids, saponins, and glycosides [9]. For instance, it was shown that the gedunin, a tetranortriterpenoid compound, extracted from mangroves displays an anti-proliferative

activity against breast cancer and ovarian cancer [10]. Similarly, crude extracts of mangroves showed to have cytotoxic effects against the cervical cancer HeLa cells [11].

The *Avicennia* is one of the true mangroves from the Acanthaceae family [12]. Globally, there are eight species of *Avicennia* [7]. *Avicennia marina* has wide geographical distribution from the Indian ocean to the western parts of the Pacific Ocean [13]. The *A. marina* is the most common species in the mangrove forests of the Persian Gulf [14, 15]. Polyphenols, presented in flavonoids, have been linked with anticancer activities of the mangrove crude extracts [16]. Polyphenolic compounds are found in abundance in mangrove species, especially in *A. marina* [17]. The *A. marina* also has some polyphenol compounds, especially flavonoids such as flavones, catechins, flavanols, and isoflavonoids are well recognized to exhibit a potent anticancer activity [16, 18]. These compounds have a wide range of therapeutic properties such as anti-microbial [19], cardiovascular protection [20], and especially anti-oxidants [21] and anti-cancer effects [22]. Evidence suggested that mangrove-derived bioactive compounds had anti-cancer effects through multiple functional mechanisms, including the modulation of cell cycle signaling [23], removal of cancerous agents [24], apoptosis [25], cell cycle arrest [26], and anti-oxidant effects [21].

In the present study, the anti-cancer effects of ethanol and ethyl acetate extracts of the *A. marina* leaves were evaluated. Phytochemical compositions were done to detect the available bioactive compounds in both extracts. Then, using experimental in vitro studies and computational modeling, the mechanism of anti-cancer activity of ethanol and ethyl acetate extracts of *A. marina* leaves on breast, ovarian and cervical cancer cell lines were determined.

Materials And Methods

Ethical approval

statements

This investigation was performed in accordance with relevant guidelines and regulations of the ethical committee of Shahid Sadoughi University of Medical Sciences (Permission number: IR.SSU.RSI.REC.1397.027).

A. marina **collection and extractions**

The leaves of *A. marina* were collected from the Bushehr subtidal region, shores of the Persian Gulf, Iran. Authentication of collected *A. marina* was done by a specialist from the Persian Gulf Marine Biotechnology Research Center, Bushehr University of Medical Science. The leaves of *A. marina* were air-dried in the dark at a room temperature and were powdered. The powders were subjected to ethanol and ethyl acetate extractions.

For ethanol extraction, 40g of dried leaves powder was dissolved in 100 mL of 70% ethanol (Merck, Germany) solvent. The extraction process was continued by shaking the mixture continuously for 3 days

at room temperature in the dark condition. Then, the ethanol was evaporated by a rotary evaporator (Laborota 4003-control, Heidolph Instruments GmbH & CO. KG, Germany). For ethyl acetate extraction, 40g of dried leaves powder was dissolved in 100 mL of ethyl acetate (Merck, Germany) for 3 days at room temperature and then ethyl acetate was evaporated by rotary evaporator (Laborota 4003-control, Heidolph Instruments GmbH & CO. KG, Germany). Then, the obtained extracts were applied for phytochemical analysis, including total phenolic and total flavonoid contents. Furthermore, the cell apoptotic and anti-proliferative effects of two extracts were tested on cancer cell lines including MCF-7, OVCAR3, and HELA and normal cells, Vero cell line.

Phytochemical analysis of A. marina extracts

Total phenolic content of A. marina extracts

The total amount of polyphenol contents of the extracts was measured by Folin–Ciocalteu method [27]. The calibration curve was determined on the basis of gallic acid (Merck, Germany). In detail, 20 mg of gallic acid was dissolved in 100 mL of 50% methanol (200 µg/mL) and then diluted to 25, 50, 75, and 100 µg/mL. Afterward, 100 µL of each concentration, 0.5 mL of Folin–Ciocalteu reagent (Merck, Germany), and 1 mL of 20% sodium carbonate (Merck, Germany) were mixed and kept in the dark for 1 h. The total amount of polyphenols was determined by ultraviolet spectrophotometry (CECIL, England) at a wavelength of 765 nm. A similar procedure was adopted for both ethanol and ethyl acetate extracts. All determinations were measured in triplicate. The results of both ethanol and ethyl acetate extracts were separately compared with the calibration curve.

Total flavonoid content of A. marina extracts

The total flavonoid content was measured by the aluminum chloride colorimetric method [28]. At first, different concentrations of quercetin (Fluorochem, United Kingdom) solutions including 25, 50, 75, and 100 µg/mL was prepared by dissolving in methanol solution and then 100 µL of the standard agent (quercetin), 0.2 mL of the aluminum chloride (Merck, Germany) solution and 0.1 mL of 33% aqueous acetic acid were added in a tube and well stirred. Finally, the materials were mixed by adding 90% ethanol until the solutions' volume reached 5 mL and it was kept at room temperature for 30 min. The total amount of flavonoid was determined by ultraviolet spectrophotometry (CECIL, England) at a wavelength of 415 nm. A similar procedure was performed for both extracts. Briefly, in the previous procedure, instead of quercetin agent, the same concentrations of ethanol and ethyl acetate extracts were separately used for flavonoid content measurement. All determinations were measured in triplicate.

GC-MS analysis of A. marina extracts

The extracts were lyophilized and were subjected to the 7890B Agilent Gas Chromatography-Mass Spectroscopy (GC-MS). Electron ionization (EI) mass spectra (scan range, m/z 50–500) were obtained using electrons with an energy of 70 eV. The filament emission was 0.5 mA. The GC separations were performed using an HP-5MS UI column (30 m × 0.25 mm i.d., film thickness 0.5 µm). Helium, as the

carrier gas, was used with the flow of 0.8 ml min^{-1} for EI. The GC oven was temperature programmed at 5°C min^{-1} from 80°C after 3 min since the sample injection and held at 250°C for 10 min. The injection port of the gas chromatograph, the transfer line, and the ion source of 5977MSD were maintained at 240°C, 250°C, and 220°C, respectively. Identification of the separated compounds was performed by comparing them with the compound data of the National Institute of Standards and Technology (NIST MS database, 2014) library. The relative percentage of each compound was measured by average peaks area in comparison with the total areas.

In vitro **analysis of** A. marina **extracts**

Cell culture

For *in vitro* study, breast cancer cell line (MCF-7), ovarian carcinoma cell line (OVCAR3), cervical cancer cell line (HeLa), and normal kidney epithelial cell line (VERO) were provided (PerciaVista Co., Iran). The cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life Technologies Co., US) supplemented with 10% fetal bovine serum (FBS, Kiazist, Iran) and 1% penicillin-streptomycin (Pen-Strep, Gibco, Life Technologies Co., US). After seeding the cell lines, the cells were incubated at 37°C and 5% CO_2.

MTT assay and calculation of cytotoxic concentration

The cell lines were seeded in 96-well cell culture plates and after 24 h, the ethanol and ethyl acetate extracts of *A. marina* leaves were separately added to all cell lines in the concentrations of 40, 80, 120, and 160 µg/mL. For the control group, the DMEM supplemented with 10% FBS and the same concentration of dimethyl sulfoxide (DMSO, Sigma-Aldrich Co., Darmstadt, Germany) was added to the wells. The samples were incubated at 37°C and 5% CO_2 for 72 h. Then, the media were removed and the cells were washed with phosphate buffer saline (PBS). For cell vitality assessment, 100 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay kit (Sigma-Aldrich Co., Darmstadt, Germany) with the concentration of 5 mg/mL and incubated for 4 h until the formation of the intracellular purple formazan crystals. After that, the supernatant was removed and DMSO was added to each well and incubated at 37°C and 5% CO_2 for 20 min. The absorbance of cells in each plate was read at a wavelength of 573 nm using an ELISA plate reader machine (BioTek, USA). This step was repeated three times. Then, 50% cell cytotoxic concentration (CC_{50}) of *A. marina* leaves for both extracts was calculated via nonlinear regression of “log (inhibitor) vs. normalized response” in Graph pad prism (v7.0a, GraphPad Software, Inc., San Diego, CA, USA).

Growth curve assay and population doubling time

The cell lines were seeded in 24-well cell culture plates at a density of 4×10^4 cells per well. Concentrations of 40, 80, 120, and 160 µg/mL of ethanol extract and ethyl acetate extract of *A. marina* leaves were prepared in 10 mL DMEM supplemented with 10% FBS and 10 mL of DMSO, respectively. The cells were treated separately with crude ethanol and ethyl acetate extracts for 24 h. After that, the number of cells in

three wells was counted every 24 hours (each time three wells/group). This procedure was repeated for 7 days, and the mean number of the cells on each day was obtained. In more detail, the culture medium supplemented with crude extracts was changed on day 3. The following formula was used to determine population doubling time (PDT): T×ln2/ln (Xe/Xb), where Xe, Xb, and T were defined as the final cell number, the initial cell number, and the incubation time in any unit, respectively.

Cell viability assessment

Trypan blue exclusion method was selected for the cell viability. In detail, the cell lines were seeded at a density of 4×10^4 cells per well. The cells were treated with 40, 80, 120, and 160 µg/mL concentrations of ethanol extract and ethyl acetate extract of *A. marina* leaves and were incubated at 37°C in the presence of 5% CO_2 for 72 h. After that, the cells were treated with 300 µL of 0.5% trypsin enzyme (Kiazist Co., Iran). Finally, 20 µL of medium contained detached cells and an equal volume of trypan blue were mixed and viable, and dead cells were counted by hemocytometer chamber. The cell viability was measured by the following formula: Cell viability (%) = (number of non-stained cells/number of total cells) × 100.

Cell cycle and apoptosis analyses by flow cytometry

The proportion of apoptotic cells was determined by flow cytometry. Based on the findings of cell MTT assay, cell viability test, and PDT assay, the most effective concentrations of the extracts were determined and used for cell cycle assay in each cell line. The cells in the logarithmic growth phase were taken for experimental intervention. After 72 h cell exposure to extracts, cells were digested, washed, and prepared as the single-cell suspension. The cell density was provided into $1–5\times10^5$ cells/mL. Then, 500 µL of binding buffer was added to suspend cells and mixed gently. Afterward, 5 µL of annexin-V-FITC and 5 µL PI were added to cells and they were incubated in the dark condition at room temperature for 15 min. The procedure was followed by l h using Flow Cytometry (FACS, Becton, Dickinson, USA). The experimental results were analyzed using BD Cell Quest software (BD Biosciences Co., USA).

Western blot analysis

Western blot analysis was done based on standard procedures with slight modifications [29]. Based on the findings of cell MTT assay, cell viability test, and PDT assay, the most effective concentrations of the extracts were determined and used for western blot analysis in each cell line. In details, after 72 h cell exposure to extracts, cells were lysed by RIPA buffer including 50 mM Tris–HCl (pH = 8.0), 0.4% Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 µg/mL leupeptin, 3 mM NaF, and 1 mM DTT at 4°C for 20 min. The lysates were centrifuged at 12000 ×g for 20 min at 4°C, and the protein concentration was measured by a Bradford protein assay. Proteins were then transferred to a microporous polyvinylidene difluoride membrane (Millipore, France). Membranes were incubated in 5% BSA (Sigma, USA) blocking buffer for 1 h at room temperature. After blocking, the membranes were incubated with the corresponding primary antibodies separately overnight at 4°C. Immunoblotting was performed with rabbit anti-Bcl-2, anti-Bcl-xl, anti-Bax, anti-caspase-1, -3, and − 7 antibodies (1:200) (Cell Signaling Technology, Danvers, MA). Membranes were washed three times (10 min each) in tween buffer before incubating with HRP-conjugated goat anti-mouse or rabbit secondary antibodies. To remove

excess antibodies, membranes were washed four times before HRP activities were detected using ECL Plus Chemiluminescence Reagent (Amersham, Chalfont, UK) according to the protocol supplied with the kit.

Computational details

Ligand and Receptor preparation

Thirty-three compounds derived from the Persian Gulf *A. marina* and five proteins were selected for the docking process. The three-dimensional (3D) structure of ligands and receptors was downloaded from the PubChem database and the Protein Database Bank (PDB), respectively. HyperChem software version 8.0.10 was used to optimize the geometry of compounds. Chimera 1.15 was used to prepare receptors. The receptor preparation process includes removing all non-standard residues, water, and original hydrogens, the addition of polar hydrogen, charges and bond orders, and capping N and C termini. Finally, the format of all outputs was converted to a suitable format for the docking process.

Generation of grid

The generation of the grid box is an important step in the docking process. To generate a grid box, the position of the active site of each receptor was found using available structures with a co-crystallized ligand for BAX, BCL-2, Caspase-1, Caspase-3 and Caspase-7 with PDB code of 4zie, 6O0K, 6f6r, 4quj and 2ql9, respectively. Then, a grid box with a dimension of 35×35×35 $Å^3$ and a spacing of 0.375 Å was generated at the active-site of each receptor using AUTOGRID.

Study of target proteins-marine derived compounds interactions

In order to study interactions of target proteins and selected compounds, the docking process was performed using Autodock Vina 1.1.2 after generating the grid box. Among different conformations suggested by software for each ligand, the best one was selected according to the binding affinity score and RMSD given by the software. In order to validate the accuracy of the docking process, the co-crystallized ligand (1,2-ethanediol, LBM, CVE, MPD, and CIT for 4zie, 6O0K, 6f6r, 4quj and 2ql9, respectively) were re-docked. The best conformations of the co-crystallized ligands were reasonably located at the original active-site with RMSD ≤ 2 Å.

Statistical analysis

IBM SPSS Statistics 26 software (SPSS for Windows, version 26, SPSS Inc, Chicago, Illinois, USA) was used for statistical analysis of data. The results were presented as mean ± standard error of mean. The measurement results received the homogeneity test of variance. Comparison between groups was done using one-way ANOVA and *post hoc* LSD test (for comparing the MTT and cell proliferation and viability assays) or chi-square test (for comparing the cell cycle analysis and flow cytometry). The $p < 0.05$ was

considered as the statistically significant difference. The graphs were drawn by Graph pad prism (v7.0a, GraphPad Software, Inc., San Diego, CA, USA).

Results

Total phenolic content of the ethanol extract of A. marina leaves was higher than the ethyl acetate extract

The ethanol extract of *A. marina* had a higher yield of extractions than the ethyl acetate extract of the *A. marina*. The total phenolic content of *A. marina* extracts was determined. The calibration curve generated from the analysis of the standard (gallic acid) was linear (Figure S1A). In detail, the ethanol extract of *A. marina* showed higher phenolic content (345 µg/ml gallic acid/0.01 g extract) than ethyl acetate extract (147.5 µg/ml gallic acid/0.01 g extract).

Total flavonoid content of the ethanol extract of A. marina leaves was higher than the ethyl acetate extract

The total flavonoid content of both ethanol and ethyl acetate extracts of *A. marina* was determined. The calibration curve generated from the analysis of the gallic acid was linear (Figure S1B). In addition, the equation and parameter were y = 0.0009x + 0.0155 and R2 = 0.9081, respectively. The results showed that the ethanol extract of *A. marina* had a higher flavonoid content (47.8 mM GAE) than ethyl acetate extract (38.6 mM GAE).

GC-MS profile of the ethanol and ethyl acetate extracts of A. marina leaves showed anti-cancer compounds

GC-MS analyses revealed several different molecules in the ethanol and ethyl acetate extracts of *A. marina* leaves (Figure S2). Sixty compounds were identified in the ethanol extract of *A. marina* leaves (Table S1). In detail, there were 26 compounds with anti-cancer and apoptotic effects based on the previous researches. In addition, 56 compounds were detected in the ethyl acetate extract of *A. marina* leaves (Table S2). Twenty of these compounds had anti-cancer and apoptotic effects based on the previous studies. The effects of both ethanol and ethyl acetate compounds on three cell lines (MCF-7, OVCAR3, and HeLa) were also evaluated in Tables S1 and S2. Moreover, other biological activities were observed in *A. marina* extracts including anti-oxidant, anti-inflammatory, antimicrobial, and anti-fungal activities are summarized in Tables S1 and S2.

MTT proliferation test of ethanol extracts of A. marina leaves had anti-proliferative effects on MCF-7

The anti-proliferative effect of 120 and 160 µg/mL concentrations of ethanol extract was observed on the MCF-7 cell line ($p < 0.05$, Fig. 1A). However, the extract had a proliferative effect on OVCAR3 cell lines at 40, 80, and 120 µg/mL concentrations ($p < 0.05$, Fig. 1B). In contrast, the ethanol extract of *A. marina* had no significant effects on the HeLa cell line at different concentrations (Fig. 1C). In addition, the ethanol extract of *A. marina* had also an anti-proliferative effect on the Vero cell line at the concentrations of 40

and 160 µg/mL ($p < 0.05$, Fig. 1D). Therefore, ethanol extract of *A. marina* leaves at 120 µg/mL had anti-proliferative effects on MCF-7 cancer cell lines comparing with normal Vero cells.

Moreover, the ethyl acetate extract had no significant effects on MCF-7 and OVCAR3 cell lines (Figs. 1E and 1F). The anti-proliferative effect of ethyl acetate extract was observed at the concentration of 160 µg/mL on the HeLa cell line ($p = 0.01$, Fig. 1G). The ethyl acetate extract had also anti-proliferative effects on the Vero cell line at the concentration of 120 and 160 µg/mL ($p < 0.05$, Fig. 1H). Therefore, ethyl acetate extract of *A. marina* leaves had no applied anti-proliferative effects on three cancer cell lines comparing with normal Vero cells.

A. marina **leaves had cytotoxic activity on MCF-7 by ethanol extract and HeLa by ethyl acetate extract**

The ethanol and ethyl acetate extracts of *A. marina* leaves potentially inhibited the viability of MCF-7 cells, OVCAR3 cells, HeLa cells, and Vero cells with CC_{50} values of 70 and 102 µg/mL, 1087 and 272 µg/mL, 189 and 67 µg/mL, and 382 and 242 µg/mL, respectively. As shown in Figs. 2A and 2B, these two extracts had significant dose-dependent inhibition on the proliferation and viability of the MCF-7 and HeLa cancer cells. The results showed that the cytotoxic activity of these extracts on MCF-7 and HeLa cancer cells was more active than OVCAR3 cancer cells and Vero normal cells.

Cancer cell count decreased by ethanol and ethyl acetate extract treatments

The ethanol extract decreased MCF-7 cell number at 7 days ($p < 0.05$, Figure S3A). Increasing the concentrations of ethanol extract 1.5 times increased the PDT of MCF-7 cells (Table 1). In addition, the ethanol extract decreased OVCAR3 cell number at five days ($p < 0.05$, Figure S3B). A dose-dependent increase of PDT to two to five times was seen on the effect of ethanol extract on OVCAR3 cell proliferation (Table 1). The ethanol extract decreased HeLa cell number from day 6 ($p < 0.05$, Figure S3C). Increasing the concentrations of ethanol extract 1.8 times increased PDT of HeLa cells (Table 1). The ethanol extract decreased Vero cell number at days 7 ($p < 0.05$, Figure S3D). A dose-dependent increase of PDT to 1.5 times was seen on the effect of ethanol extract on the Vero cell proliferation (Table 1).

Table 1
Mean of population doubling time of MCF-7, OVCAR3, HeLa and Vero cell lines after exposure to different concentrations (µg/mL) of ethanol and ethyl acetate extracts of Avicennia marina leaves

Extracts	Concentrations (µg/mL)	Population doubling time (PDT) (days)			
		MCF-7	OVCAR3	HeLa	Vero
Ethanol	0	1.1	2.5	1.5	1.1
	40	1.3	6	1.7	1.6
	80	1.3	4.2	2	1.6
	120	1.4	10.6	2	1.6
	160	1.6	5	2.8	1.6
Ethyl acetate	0	1.1	2.5	1.4	1.1
	40	1.5	3.4	1.5	1.3
	80	1.6	2.8	1.6	1.5
	120	1.4	2.7	2.9	1.4
	160	2	6.8	2.8	1.7

Moreover, the ethyl acetate extract decreased MCF-7 cell number at 5 days ($p < 0.05$, Figure S3E). Increasing the concentrations of ethyl acetate extract 1.8 times increased PDT of MCF-7 cells (Table 1). In addition, the ethyl acetate extract decreased OVCAR3 cell number at five days ($p < 0.05$, Figure S3F). A dose-dependent increase of PDT to 2.7 times was seen on the effect of ethyl acetate extract on the OVCAR3 cell proliferation (Table 1). The ethyl acetate extract decreased HeLa cell number from days 7 ($p < 0.05$, Figure S3G). Increasing the concentrations of ethyl acetate extract 2 times increased PDT of HeLa cells (Table 1). The ethyl acetate extract decreased Vero cell number at 3 days ($p < 0.05$, Figure S3H). A dose-dependent increase of PDT to 1.5 times was seen on the effect of ethyl acetate extract on the Vero cell proliferation (Table 1).

A. marina **ethanol extracts reduced the viability of OVCAR3 and HeLa cells**

160 µg/mL ethanol extract of *A. marina* leaves decreased OVCAR3 and HeLa cells viability from day 2 ($p < 0.05$, Figs. 3B and 3C). The 160 µg/mL ethyl acetate extract of *A. marina* leaves decreased the viability of OVCAR3, HeLa and Vero cells from days 6, 5, and 5, respectively ($p < 0.05$, Figs. 3F and 3G).

Cell cycle analysis of HeLa cell lines showed an increase of dead cells

Based on the findings of the cell MTT assay, PDT assay, and cell viability test, the most effective concentrations of the extracts were determined and used for cell cycle assay (Fig. 4). The MCF-7 cell line was treated with 120 µg/mL concentration of ethanol extract of *A. marina* leaves that 2-times increased

the number of cells in the S phase. The OVCAR3 cell line was treated with 160 µg/mL concentration of ethyl acetate extract. The HeLa cell line was treated with 120 µg/mL concentration of ethyl acetate extract. The proportion of dead cells increased in HeLa cells after treatment with ethyl acetate extract of *A. marina* leaves ($p < 0.05$, Fig. 4l).

Cell apoptosis analysis of MCF-7, OVCAR3, and HeLa cell lines showed increase of apoptotic cells by A. marina leaves extracts

Based on the findings of the cell MTT assay, PDT assay, and cell viability test, the most effective concentrations of the extracts were determined and used for cell apoptosis assay (Fig. 5). The MCF-7 cell line was treated with 120 µg/mL concentration of ethanol extract of *A. marina* leaves. The OVCAR3 and HeLa cell lines were treated with 160 µg/mL and 120 µg/mL of ethyl acetate extract, respectively. After treating the HeLa, MCF-7, and OVCAR3 cell lines, the proportion of apoptotic cells increased ($p < 0.05$, Figs. 5G-5l).

Western blot analysis showed an increase of BAX, caspase-1, -3, and − 7 expressions in MCF-7, OVCAR3, and HeLa cell lines by A. marina leaves extracts

The MCF-7 cell line was treated with 120 µg/mL concentration of ethanol extract of *A. marina* leaves. The OVCAR3 and HeLa cell lines were treated with 160 µg/mL and 120 µg/mL of ethyl acetate extract, respectively. Expressions of BAX, cleaved-caspase-1, -3, and − 7 increased in MCF-7, OVCAR3, and HeLa, and cell lines after treatment (Fig. 6). However, expressions of BCL-2, pro-caspase-1, -3, and − 7 decreased in MCF-7, OVCAR3, and HeLa cell lines after treatment (Fig. 6).

Five bioactive molecules in A. marina leaves extracts had the highest affinity to apoptotic peptides

Thirty-three compounds derived from ethanol and ethyl acetate extracts of the *A. marina* leaves (Table 2) have been selected based on the previous studies as anti-cancer compounds (Tables S1 and S2) to study their binding affinity to apoptotic proteins BAX, BCL-2, caspase-1, -3, and − 7 through the docking process. Though, the ligand-protein complexation is controlled by conformations and intermolecular interactions such as electrostatic and Van der Waals forces [30]. The most stable complex has the lower negative energy or binding affinity, ΔG [U total in kcal/mol], as the lower negative energy indicates the more favorable ligand-protein interaction. The results of the docking process are shown in Table 2. According to Table 2, the binding affinity is in the range of -3.6 to -10.8 kcal/mol. Among selected compounds, ergosta-5,22-dien-3-ol, acetate shows the best affinity for caspase-7. Stigmasterol shows the best affinity for BAX. Beta amyrin shows the best binding affinity for BCL-2, caspase-1 and caspase-7. Moreover, it was found that three compounds of alpha amyrin, beta amyrin and cholesta-22, 24-dien-5-ol,4,4-dimethyl have the same affinity to caspase-3. The intermolecular interactions of these compounds are shown in Fig. 7.

Table 2
The results of the docking process of anti-cancer compounds in ethanol and ethyl acetate extracts of *Avicennia marina* leaves and their interactions with apoptotic peptides in cancer cells

Ligands	Binding affinity (Kcal/mol)				
	BAX	BCL-2	Caspase 1	Caspase 3	Caspase 7
(R)-3-hydroxydecanoic acid	-4.8	-4.8	-4.9	-4.3	-5.6
2,4-Di-Tert-Butylphenol	-6.4	-6	-5.7	-4.8	-7.3
2-Tridecanol	-4.8	-5	-4.5	-4.1	-5.6
4-Butoxy-2-methyl-2-pentanyl acetate	-4.6	-5.2	-4.7	-4.5	-6.1
9,12-Octadecadienoyl chloride	-5.6	-5.8	-4.2	-3.9	-6.4
Alpha amyrin	-8.4	-8.4	-7.3	-6.7	-8.3
Beta amyrin	-7.7	-9.5	-7.6	-6.7	-6.6
Betulin	-7.8	-7.4	-6.5	-6.2	-8.1
Cholesta-22, 24-dien-5-ol,4,4-dimethyl	-8.4	-8.2	-6.9	-6.7	-10.5
Cyclohexanol 1-methyl-4-(1-methylethyl)-	-5.3	-6	-5.4	-4.7	-5.8
Decanoic acid	-4.7	-4.8	-4.5	-3.9	-5.3
Dihydrocarveol	-5.3	-5.8	-5.2	-4.3	-6.1
Dodecane	-4.5	-4.6	-4.3	-4.2	-5.2
Ergosta-5,22-dien-3-ol, acetate	-8.7	-8.1	-6.8	-6.2	-10.8
Ethyl oleate	-5	-5.7	-4.4	-4.5	-6.7
Gamma-Sitosterol	-5	-8.5	-6.2	-6	-10.1
Hexadecane	-4.8	-4.7	-3.6	-3.7	-6.1
Hexadecanoic Acid 2-Hydroxy-1-(Hydroxymethyl) Ethyl Ester	-5.6	-5.4	-4.7	-4.1	-6.2
Levoglucosan	-5.3	-4.8	-4.2	-4.2	-4.9
Linoleic acid	-5.6	-5.6	-4.8	-4.2	-6.7
Lupeol	-8.3	-7.9	-6.8	-6.5	-8.4
Myristic acid	-5.1	-5	-4.8	-4	-6.1
Myristoleic acid	-5.2	-5.4	-4.8	-3.9	-6.4
Norspermidine	-3.3	-3.6	-4	-3.4	-4.1

Ligands	Binding affinity (Kcal/mol)				
	BAX	BCL-2	Caspase 1	Caspase 3	Caspase 7
Octadecanoic acid	-5.1	-5	-4.6	-4	-6.3
Octadecanoic acid, ethyl ester	-5.2	-5.1	-4.4	-4.1	-6.1
Palmitic acid	-5.1	-5	-4.9	-4	-6.1
Pentadecanoic acid	-5.1	-5.3	-4.1	-3.6	-6.2
Phenylmethyl ester	-6	-5.4	-4.5	-4	-7.7
Phytol	-5.9	-5.8	-4.5	-4.5	-6.9
Squalene	-7.3	-7.8	-4.7	-4.4	-9.5
Stigmasterol	-8.8	-8.4	-6.6	-6	-10
Vitamin E	-7.5	-5.8	-5.8	-4.5	-9.3

Discussion

Ethanol and ethyl acetate extracts of A. marina leaves have anti-cancer compounds

Thirty-three compounds of both ethanol and ethyl acetate extracts of *A. marina* had anti-cancer biological effects. Previous studies showed that the linoleic acid compound had anti-cancer activity on MCF-7, OVCAR3 and HeLa cell lines [31–33]. Other anti-cancer compounds of *A. marina* extracts have shown in Tables S1 and S2. These results suggest the potent anti-cancer and cytotoxic effects of ethanol and ethyl acetate extracts of *A. marina* on cancer lines. The results of the current study demonstrated that both ethanol and ethyl acetate extracts of *A. marina* had polyphenol and flavonoid contents. Consistent with our results, it has been shown that one of the phytochemical compounds of *A. Marina* is polyphenols such as flavonoids [34]. In detail, the ethanol extract had a higher phenolic and flavonoid content than ethyl acetate extract. Previous study also showed the same result in which the ethanol extract of *Sonneratia apetala*, another species of mangrove, had higher polyphenol content [35]. The previous study has shown that the polyphenol content and especially flavonoid content of foods are related to decreasing risk factors of some diseases such as cancer [36]. Polyphenols can inhibit cell growth and induce apoptosis through downregulation of surviving gene expression, a member of the inhibitor apoptosis protein family (IAP) that inhibits caspases and blocks cell death and Bcl-2 [37]. Polyphenols upregulate BAX expression in cancer cell lines leading to a pro-apoptotic situation which was accompanied by the upregulation of p21 and p27 leading to cell death due to activation of caspase-3 and − 9 [37]. It was shown that the *A. marina*, a mangrove species, has anti-cancer and anti-proliferative activities on different cell lines [38]. Therefore, using experimental and computational modeling of anti-cancer activity, we showed mechanisms of these compounds on cancer cell apoptosis or cell cycle arrests.

Ethanol extract of A. marina leaves induced cell cycle arrest in MCF-7 breast cancer cell lines

The MTT proliferation assay and cell count analysis showed that ethanol extract of *A. marina* leaves decreased proliferation of MCF-7 (ER^+, PR^+) breast cancer cell line. Consistent with our findings, the ethyl acetate extract of *A. marina* leaves reduced proliferation of AU565 (ER^-, PR^-) and BT483 (ER^+, PR^+) breast cancer cell lines [34]. Cell cycle analysis of MCF-7 with ethanol extracts showed an increase in cell population in S-phase. However, the cell viability of MCF-7 did not alter by extracts of *A. marina* leaves. In the cell cycle, there are four main phases; G_1-phase in which the cell prepared for chromosome replication. At this stage, the chromosomes are 2n. At the next stage, cells go to S-phase in which the chromosomes replicated and became 4n. After that, the cells undergo G_2 and mitosis (M) phases in which they are prepared for cell division. The G_0-phase is another part of the cell cycle. At the G_0-phase, the cell cycle will arrest and the cell division and chromosome replication will stop [39]. In contrast with our findings, AU565 and BT483 cells were treated with ethyl acetate extract of *A. marina* leaves showed an increase in percentages of cells in the sub-G_1 phase [34]. Previous studies showed that the mangrove extract decreased cell proliferation at different concentrations [34, 38, 39]. Previous studies on the anti-cancer effects of mangrove extracts could be different due to the type of the cells, type of the extract, and different concentrations of the extract [38, 40]. By the way, the current study showed that the ethanol extract of *A. marina* leaves induced its anti-proliferative effects by cell cycle arrest in MCF-7 breast cancer cell lines.

Ethyl acetate extract of A. marina leaves induced apoptosis in HeLa cervical cancer and OVCAR3 ovarian cancer cell

Cell viability of HeLa and OVCAR3 decreased by ethanol and ethyl acetate extracts of *A. marina* leaves. Furthermore, ethyl acetate extracts of *A. marina* leaves increased apoptosis of HeLa and OVCAR3. One of the essential parts of the cell cycle is apoptosis, which controls cell proliferation. It stops abnormal cells that contained irreversible damaged DNA [41]. In line with current results, previous studies on *A. marina* have shown that the different concentrations of *A. marina* extracts could induce apoptosis and increase cell population at late- and early-apoptosis, and necrosis quadrant increase [38, 42]. In the current study, Bax, cleaved caspase-1, cleaved caspase-3, and cleaved caspase-7 as pro-apoptosis effectors were increased after cell treatment and the Bcl-2, caspase-1, caspase-3, and caspase-7 as anti-apoptotic effectors decreased. B-cell lymphoma 2 protein (Bcl-2) family has a main role in the intrinsic apoptosis pathway. The Bcl-2 proteins include two subgroups: pro-apoptosis and anti-apoptosis groups. The pro-apoptosis group contained proteins such as Bax, Bcl-Xs, Bak, Bad, etc [39, 43]. On the other hand, the anti-apoptosis proteins contained Bcl-2, Bcl-W, Bcl-XL, cleaved caspase-7, etc. [39, 43, 44]. In normal cells, there are some balances between the pro-apoptosis (Bax) group and the anti-apoptosis (Bcl-2) group. However, in cancer cells, the Bcl-2 level increases and disturbs the balance. This disturbance causes inhibition of apoptosis [39, 43]. Previous studies have shown that the most important genes involved in apoptosis are Bcl-2, caspase-3, and p53 [45]. In addition, a previous study showed that inhibition of caspase-1 could induce cell death and modulate Bcl-2 expression [46]. These results demonstrated that

treatment of the HeLa and OVCAR3 cell lines via *A. marina* ethyl acetate extract induced pro-apoptotic pathways and suppressed anti-apoptotic pathways (Fig. 8). It was shown that the *A. marina* extract triggered cell apoptosis using p53 and Bcl-2 genes that caused DNA fragmentation and cell death [42].

According to the result of the computational study, among all ligands investigated through molecular docking, five ligands (ergosta-5,22-dien-3-ol, acetate, stigmasterol, alpha amyrin, beta amyrin, and cholesta-22, 24-dien-5-ol,4,4-dimethyl) showed the highest affinity to target receptors. As illustrated in Fig. 7, several intermolecular interactions including van der Waals interactions, hydrophobic interactions, and hydrogen bonds are involved in controlling the binding of ligands to receptors. Among all of these ligands, beta amyrin, which is a triterpenoid, showed the best affinity to three receptors of BCL-2, caspase-1, and caspase-3. However, its affinity for binding to BCL-2 is more than other targets. Accordingly, beta amyrin can be introduced as one of the most effective anti-cancer contents of the *A. marina* extract. This result is in accordance with those, which have been reported on the inhibitory properties of triterpenoids against the growth and proliferation of different cancer cells such as breast and prostate cancer cells [47–49]. In addition, as the smallest value of the HOMO/LUMO energy gap shows the high biological activity of molecules [50], the βHOMO/βLUMO gap energy (-1.057eV) reported for beta amyrin demonstrate good biological activity of this substance [51].

Conclusions

Ethanol and ethyl acetate extracts of *A. marina* showed to have anti-cancer biological activity on MCF-7, HeLa, and OVCAR3 cell lines. The ethanol extract induced its effects by cell cycle arrest in MCF-7. Furthermore, in HeLa and OVCAR3 cell lines, *A. marina* ethyl acetate extract triggered pro-apoptosis effectors of cancer cells and decreased expression of anti-apoptosis effectors; that's how it induces apoptosis in these cancer cell lines.

Abbreviations

3D, three-dimensional

A. marina, *Avicennia marina*

CC_{50}, 50% cell cytotoxic concentration

DMEM, Dulbecco's Modified Eagle Medium

DMSO, dimethyl sulfoxide

EI, Electron ionization

FBS, fetal bovine serum

GC-MS, Gas Chromatography-Mass Spectroscopy

HeLa, cervical cancer cell line

MCF-7, breast cancer cell line

MTT ,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

OVCAR3, ovarian carcinoma cell line

PDT, population doubling time

Pen-Strep, penicillin-streptomycin

VERO, normal kidney epithelial cell line

Declarations

Ethics and Consent to Participate

This investigation was performed in accordance with relevant guidelines and regulations of the ethical committee of Shahid Sadoughi University of Medical Sciences (Permission number: IR.SSU.RSI.REC.1397.027).

Consent to Publish

Not applicable.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Funding

This work was supported by a grant from Shahid Sadoughi University of Medical Sciences.

Authors' contributions

A. T., M. A., N. B., M. M., M. U., and I. N. conceived and designed the format of the manuscript. A. A., A. K., A. T., N. B., M. Z., G. M., A. B. 1., M. K., H. A., A. D., and A. B. 2. collected the data, and drafted and edited the manuscript. A. T., N. B, A. A., and A. K. computational and statistical analysis. N. B., A. T., A. K., and A. A. drew the Figures and Tables. All the authors reviewed the manuscript and all of them contributed to the critical reading and discussion of the manuscript. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

Not applicable.

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Figures

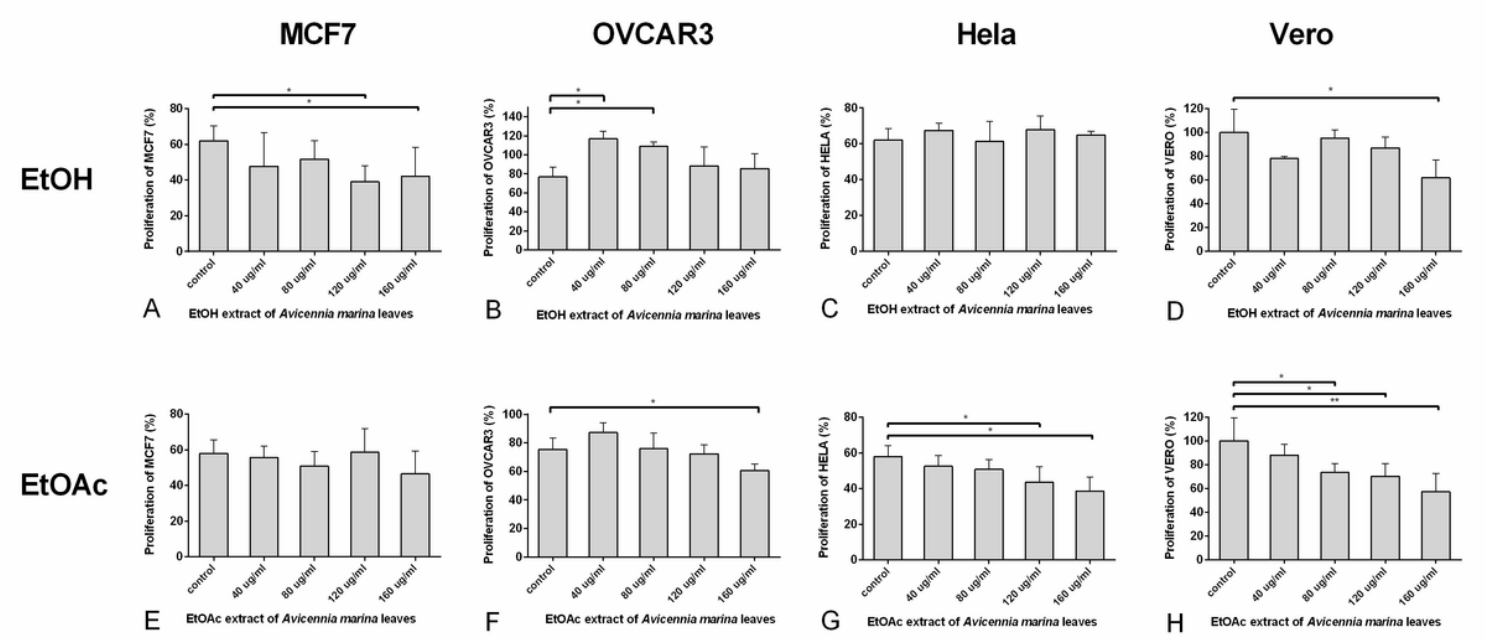

Figure 1

MTT proliferation assay of different concentrations of ethanol and ethyl acetate extracts of Avicennia marina leaves on MCF-7, OVCAR3, HeLa and Vero cell lines. Lines above the columns showed differences between treatments and control (*p<0.05 and **p<0.01).

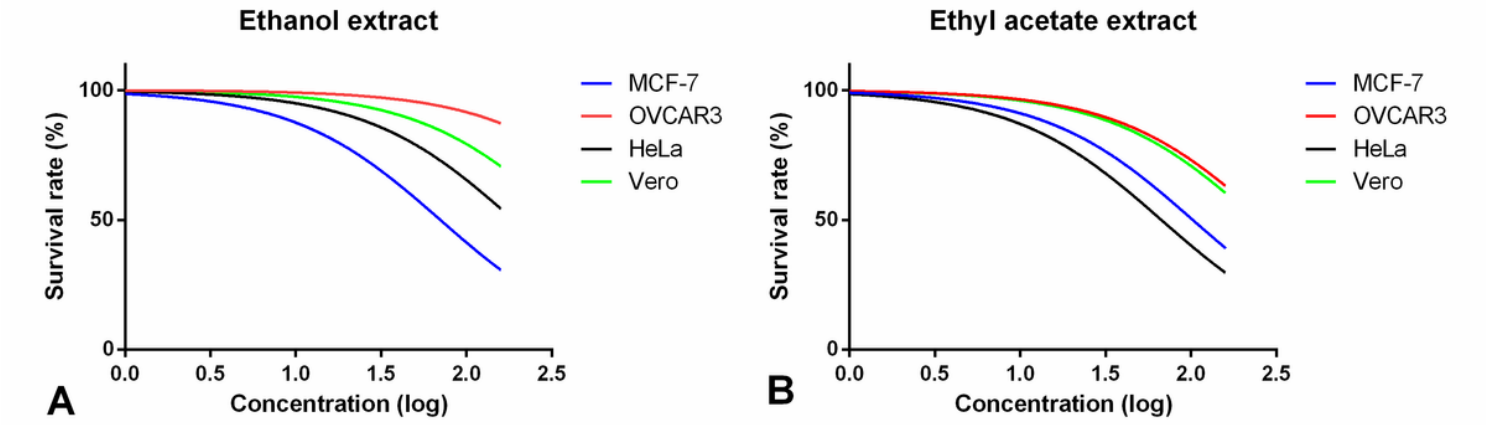

Figure 2

Cytotoxic activity of A) ethanol and B) ethyl acetate extracts from Avicennia marina against MCF-7, OVCAR3, HeLa and Vero cell lines.

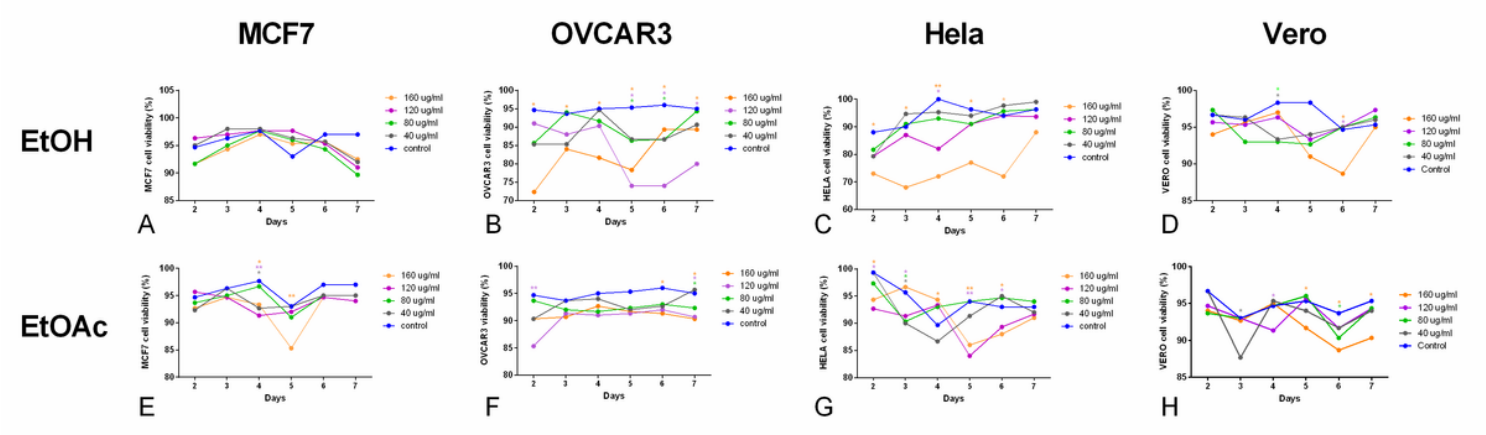

Figure 3

Cell viability assay of different concentrations of ethanol and ethyl acetate extracts of Avicennia marina leaves on MCF-7, OVCAR3, HeLa and Vero cell lines during seven days. Stars above the dots showed differences between treatments and control (*p<0.05 and **p<0.01).

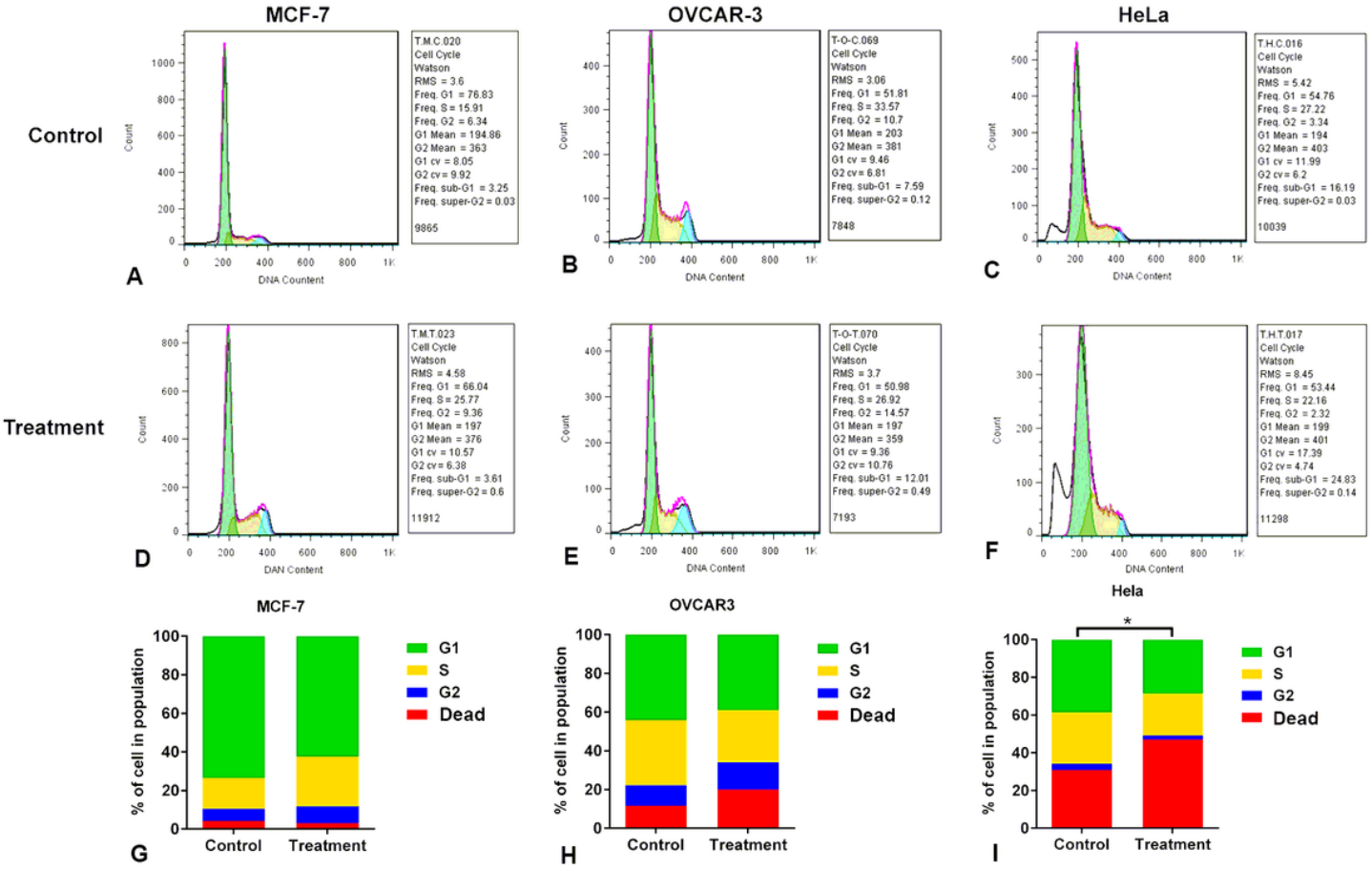

Figure 4

Cell cycle assay of extracts of Avicennia marina leaves. A) MCF-7 cell line with no treatment, B) OVCAR3 cell line with no treatment, and C) HeLa cell line with no treatment. D) MCF-7 cell line treated with 120 µg/mL concentration of ethanol extract, E) OVCAR3 cell line treated with 160 µg/mL concentration of

ethyl acetate extract, and F) HeLa cell line treated with 120 µg/mL concentration of ethyl acetate extract. G, H, and I) Comparisons of percent of cell stages in populations between control and treatment. The line above the columns shows significant differences between groups (P<0.05).

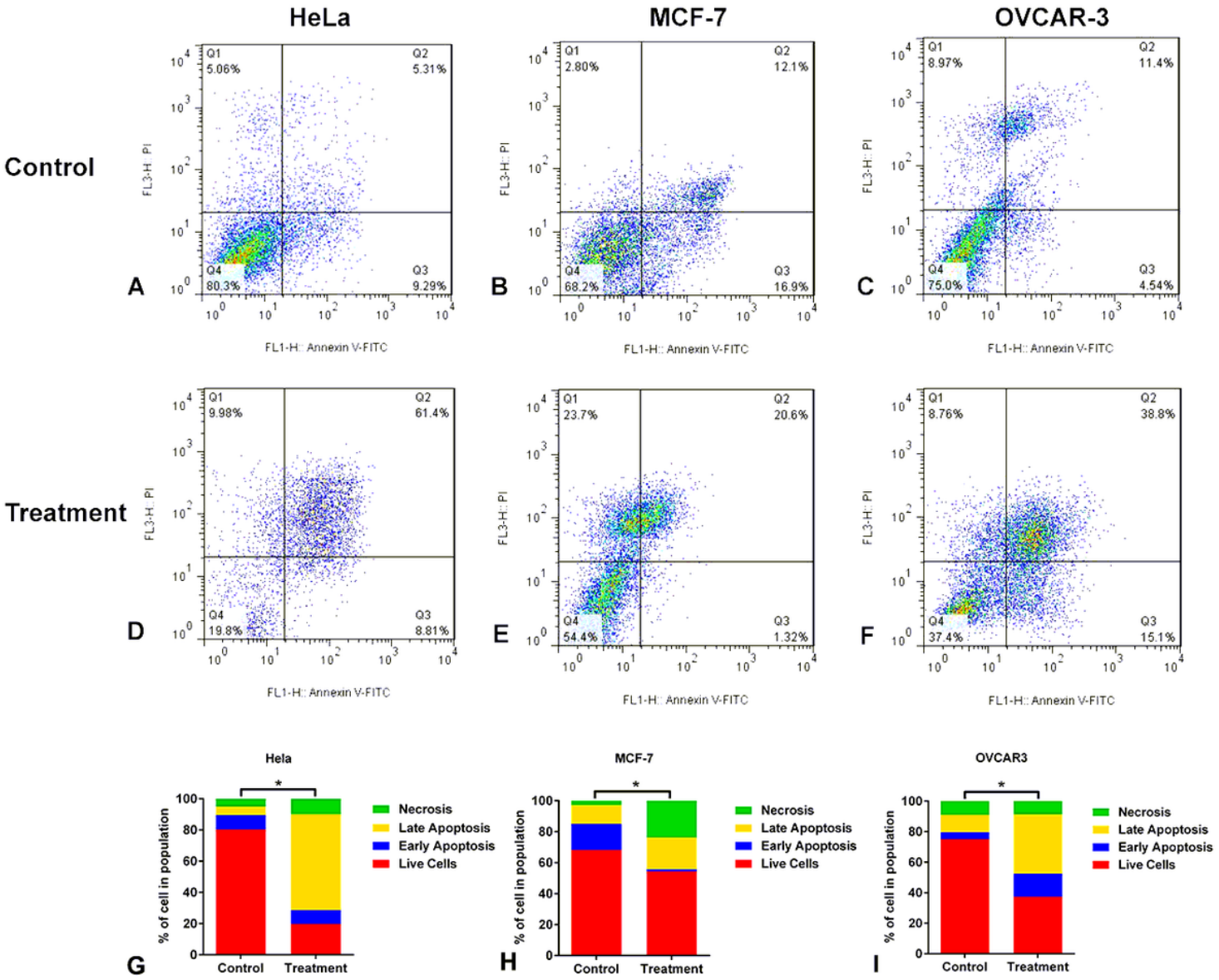

Figure 5

Cell apoptosis flow cytometry assay of HeLa, MCF-7, OVCAR3 cell lines after exposure to extracts of Avicennia marina leaves. A) HeLa, B) MCF-7, and C) OVCAR3 cell lines with no treatment as the control group. D) HeLa cell line treated with 120 µg/mL concentration of ethyl acetate extract. E) MCF-7 cell line treated with 120 µg/mL concentration of ethanol extract. F) OVCAR3 cell line treated with 160 µg/mL concentration of ethyl acetate extract. G, H, and I) Comparisons of percent of cell apoptosis in populations between control and treatment. The line above the columns shows significant differences between groups (P<0.05).

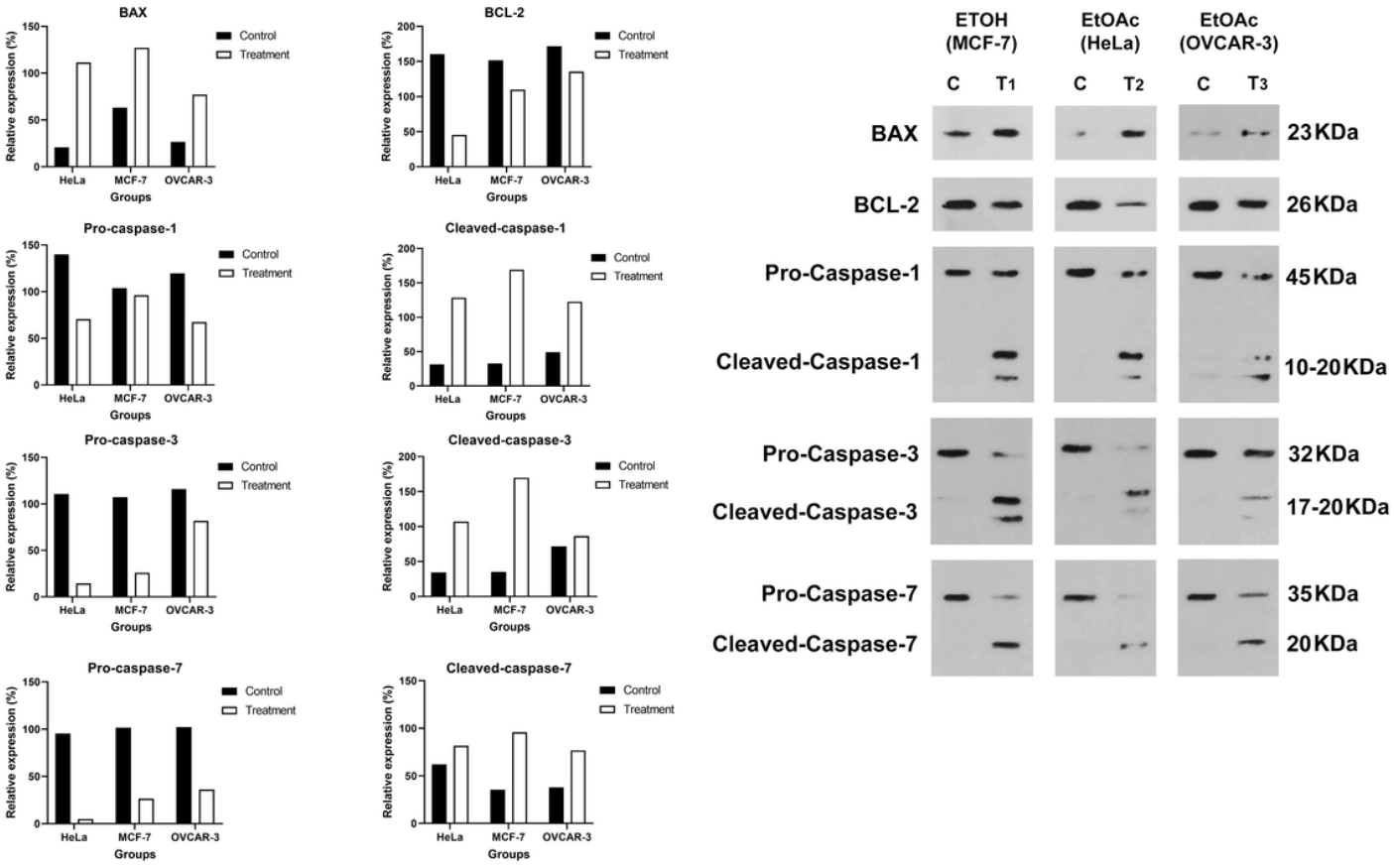

Figure 6

Expression of apoptosis proteins after treating of HeLa, MCF-7 and OVCAR3 cell lines with ethanol and ethyl acetate extracts of Avicennia marina leaves. The protein bonds of the MCF-7 cell line treated with 120 µg/mL concentration of ethanol extract (ETOH) extract, HeLa cell line treated with 120 µg/mL ethyl acetate (EtOAc) extract and OVCAR3 cell line treated with 160 µg/mL EtOAc extract of A. marina could be observed on western blot gel. C, control and T, treatment. The grouping of blots cropped from different gels.

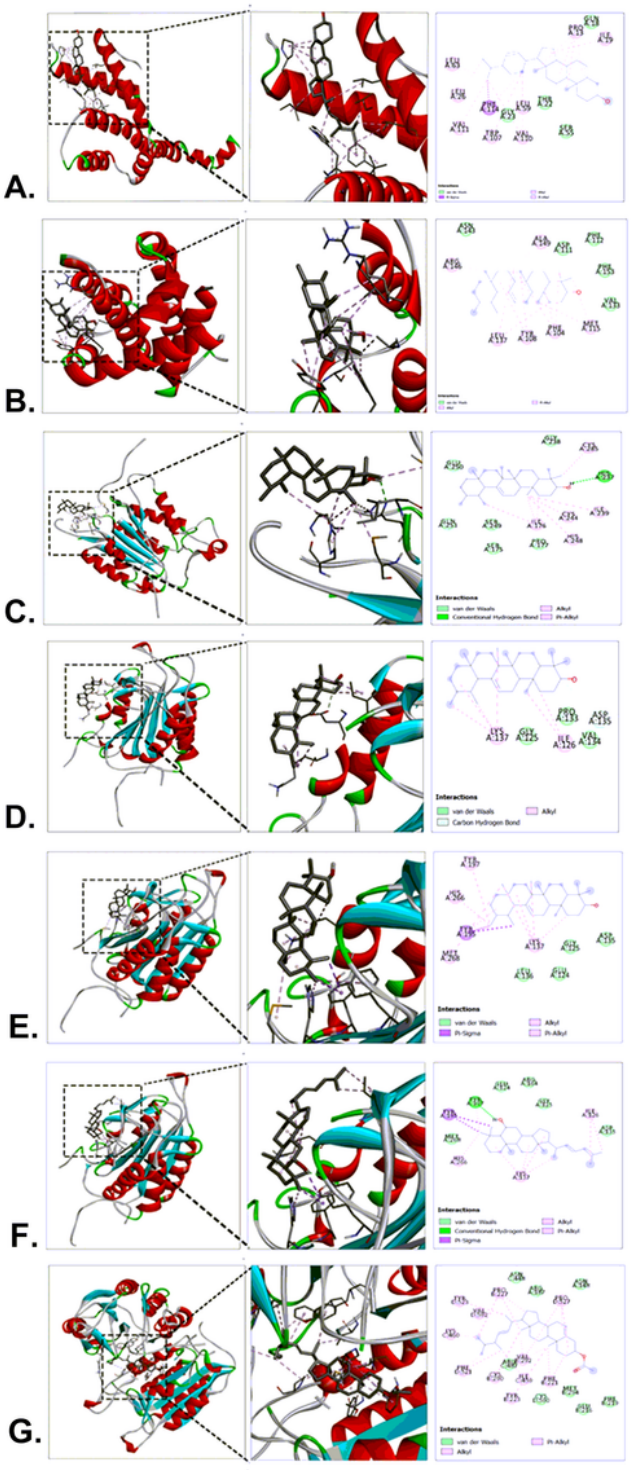

Figure 7

Computational modeling of interaction of bioactive compounds in ethanol and ethyl acetate extracts of Avicennia marina leaves on apoptosis peptides demonstrating by the three-dimensional plot of the binding sites and the two-dimensional plot of interactions. Interactions of A) BAX with stigmasterol, B) BCL-2 with beta amyrin, C) caspase-1 with beta amyrin, D) caspase-3 with alpha amyrin, E) caspase 3

with beta amyrin, F) caspase-3 with cholesta-22, 24-dien-5-ol,4,4-dimethyl, and G) caspase-7 with ergosta-5,22-dien-3-ol, acetate.

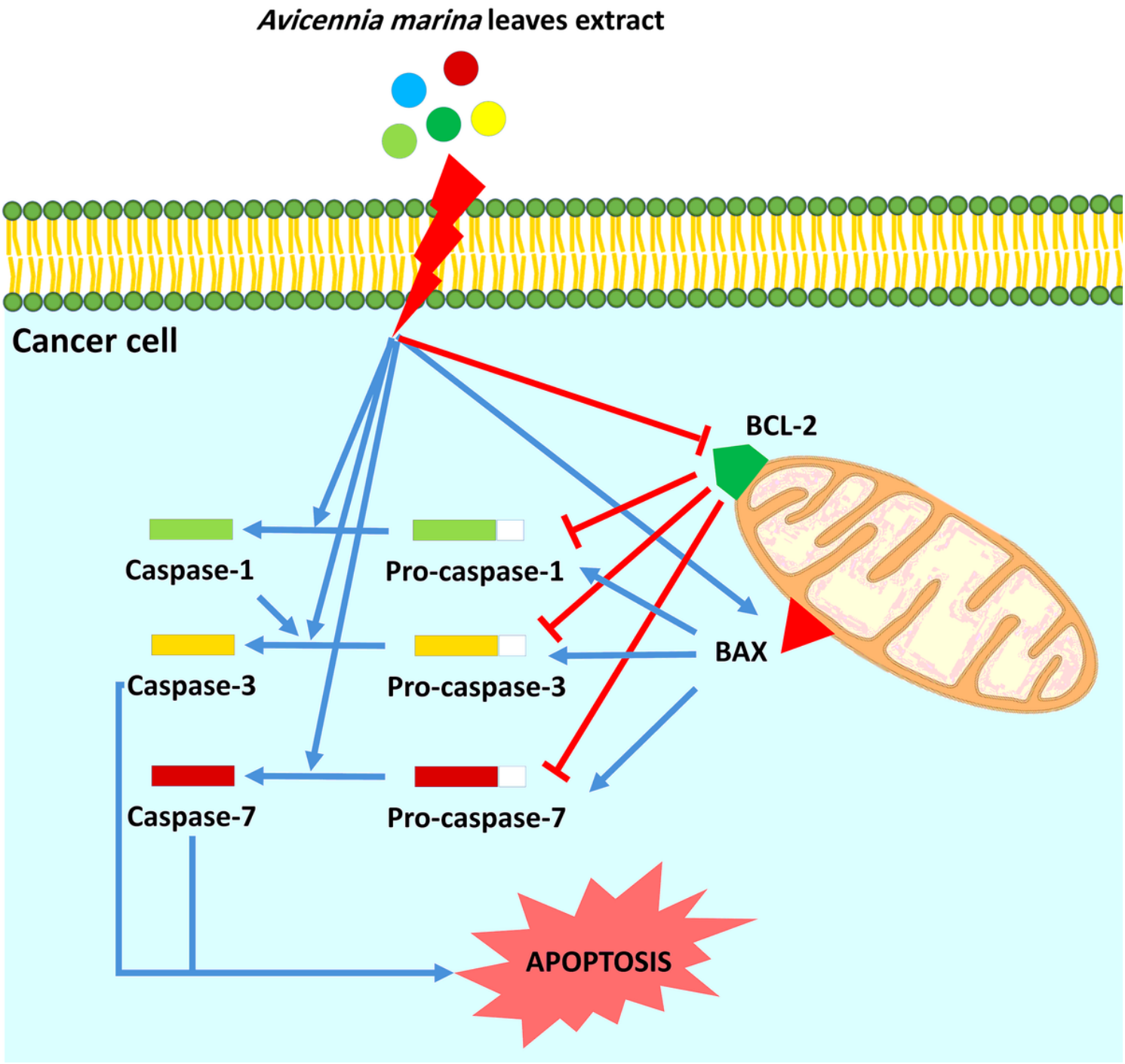

Figure 8

Avicennia marina leaves extract induces apoptosis pathway in HeLa, MCF-7 and OVCAR3 cell lines. Small colorful dots, red lighting, blue arrows and red hammer-head lines represent the A. marina extract five bioactive molecules were detected by docking technique, A. marina extract extract-induced stimulus, direct reactions and each inhibiting reaction, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile.docx
- Supplementaryfile2westernblot.docx