ASSESSMENT OF ANTICANCEROGENIC EFFECT OF *Tamarix nilotica* ON HUMAN LUNG CANCER CELL LINE

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Tamaricaceae is a small family including four genera and 120 species (Trease and Evans, 2002). In Egypt, different parts of *Tamarix nilotica* are used; the leaves and young branches are cooked for oedema of spleen and mixed with ginger for uterus infections, while the bark, when boiled in water with vinegar is used as lotion against lice (Boulos, 1983). Antioxidant and hepatoprotective activities were evaluated for total flower extract (Abouzid *et al*., 2008; Abouzid and Sleem, 2011). Phenolic compounds are known to have antioxidant activity (Tepe *et al*., 2006).

Orabi *et al*. (2010) reported that *T. nilotica* grown in Egypt exhibited significant host-mediated antitumor activities against sarcoma-180 in mice and strong cytotoxic effects with higher tumor specificity against four tumor cell lines. It showed a selective cytotoxic potential against liver (HUH-7), colon (HCT-116), lung and breast (MCF-7) carcinoma cell lines (Abouzid and Sleem 2011).

Medicinal plants are known to induce the process of apoptosis which is involved with different tumor suppressor genes including *p53*. The *p53* gene induces apoptotic cell death by direct or indirect by change expression of Bcl-2 family of proteins (Reed, 1999; Levine *et al*., 1991). The *Bcl-2* gene is an anti-apoptotic gene that suppresses initiation steps of apoptosis via inhibition of the pro-apoptotic proteins (Youle and Strasser 2008). The *p53* gene may modulate susceptibility of cells to apoptosis by down regulation of *BCL-2* and by causing up-regulation of *BAX* (Choi *et al*., 2000).

This study aimed to assess the potential anticarcinogenic effect of crude extracted from the halophyte Egyptian medicinal plant; *Tamarix nilotica*, on growth of lung human cancer compared with normal cell lines. The possible underlying antiproliferation mechanisms were evaluated by studying the effect of *T. nilotica* on apoptosis, cell cycle arrest, and apoptosis-related genes of the lung cancer cell line.

MATERIALS AND METHODS

Plant collection

Tamarix nilotica plant was collected from Wadi Gharandal, South Sinai,

Egypt in March 2015 by Desert Research Center (DRC).

Preparation of plant extract

The method and protocol followed by Bakr, *et al.* (2013) were used with modification as follow: Fresh leaves were washed; air dried at room temperature and were milled into powder. Extract was obtained by shaking at a ratio of 250 g powder in 500 ml of Ethyl acetate (piochem). It was left for 24 hr at 37°C for 3 days, then filtered through filter paper, evaporated under vacuum and the yield about (2.43 g) of (one kg) powder. After drying under vacuum, the extracts were then dried and finally placed in glass vials and re-suspended in corn oil until use.

In vitro cytotoxicity by Neutral red assay (NR assay)

In vitro neutral red cytotoxicity assay described by Fotakis and Timbrell (2006) was carried out. Culture medium containing five concentrations of *T. nilotica* extract (10, 25, 50, 100 and 200 µg/ml) in triplicate and medium without extract was considered as untreated control. The washed dye-medium was isolated and the plates with formol-calcium. A volume of 500 µl of acetic acid-ethanol (one ml glacial acetic acid in 100 ml 50% ethanol) was added and the plates were saved for 15 min at room temperature. The plates were then shaked gently for few seconds, so that complete dissolution was achieved. The absorbance of the extracted dye was measured using spectrophotometric reading (Spectra max 190-Molecular devices) at 540 nm filter. The average of three measurements for each concentration was determined. The percentage viability of each concentration of extract which reflects half the maximum concentration of the cell proliferation (IC50) was estimated. Cytotoxicity assay was measured as OD (optical density) at 540 nm. Dose-response curves were plotted, and the half maximum concentration 50% inhibitory concentrations of *T. nilotica* (IC50) were calculated by Graph Pad Prism software program.

Cell cycle analysis by propidium iodide (PI) staining using flow cytometry

To assess the effect of crude extract of *T. nilotica* on the cancerous cell line (A549) and normal human cells lines (Wi38) as control. The cell lines were treated with plant extract and digested with warm Trypsin-EDTA + warm PBS-EDTA (0.25%) (500 µl + 500 µl) and incubated for 10 minutes at 37°C. The mixture was centrifuged at 450 rpm for 5min, and then the supernatant was carefully removed. The mixture was washed twice in warm PBS and the cell pellet was resuspend in 500 µl warm PBS, centrifuged and supernatant was removed. A volume of 150 µl PBS + 350µl Ice-cold absolute ethanol (final cons. of EtOH is 70%) was added, mixed with a pipette then vortexed several times, and incubated at 4°C for 1 hour to fix the cells. To remove ethanol, the mixture was centrifuged at 350 rpm for 10 minutes and then the supernatant was carefully removed. The mixture was

washed twice in warm PBS and the cell pellet was re-suspend in 500 µl warm PBS, centrifuged and the supernatant was removed. The pellet was re-suspended in 100 µl PBS and was stored at 4°C for up to 4 days. In darkness, the cells were stained with 100 µl of PI solution + 50 µl RNase A solution (100 µg/ml), and incubated in darkness for 30-60 min. The stained cells were read in Attune flow cytometer (Applied Bio-system, US).

Analyses of apoptotic cells by AO/EB double staining:

The different morphological changes of the cells were examined after staining with ethidium bromide/acridine orange (AO/EB) by fluorescence microscopy. The A549 cells (4×10^4 cells/ml) cultured in the presence or absence of the extract were collected and washed in cold PBS. Then the AO/EB solution (1:1v/v) was added to the cell suspension at a final concentration of 100 µg/ml and then they were incubated at room temperature for 5 min. The stained cells were examined under a fluorescent microscope.

Expression of apoptosis related genes using qRT-PCR

Total RNA was isolated from A549 cells using Gene JET RNA Purification Kit (Thermo Scientific, #K0731, USA) according to the manufacturer's protocol. Total RNA (5 µg) was reversing transcribed using Revert Aid H Minus Reverse Transcriptase (Thermo Scientific, #EP0451, USA) to produce cDNA. The cDNA was used as a template to determine the relative expression of the apoptosis-related genes using StepOnePlus real time PCR system (Applied Biosystem, USA). The primers were designed by Primer 5.0 software (Table 1). The housekeeping gene β-actin was used as a reference to calculate fold change in target gene expression. A 25-µl PCR mix was prepared by adding 12.5 µl of 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, # K0221, USA), 2 µl of cDNA template, 1 µl forward primer, 1 µl reverse primer, and 8.5 µl of nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, 40-45 cycles of amplification of DNA (denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec). At the end of the last cycle, the temperature was increased from 63 to 95°C for melting curve analysis. The cycle threshold (Ct) values were calculated for target genes and the housekeeping gene, and relative gene expression was determined using $2^{-\Delta\Delta CT}$ method

Statistical analysis

For statistical analysis of cell lines data, multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the LSD test for post hoc analysis. Statistical significance was accepted at a level of $P < 0.05$. Data were analyzed using SPSS (version 11; Chicago, IL, USA). In Expression of apoptosis related genes were expressed as means ± standard error (SE). The obtained data were subjected to one way analysis of

variance (ANOVA) according to (Snedecor and Cochran, 1980) using SPSS 18.0 software. Least significant differences were used to compare between mean of treatments according to Duncan test at probability of 5%.

RESULTS AND DISCUSSION

Cytotoxic activity on A549 and Wi38 cells lines by Neutral red assay

Data presented in Fig. (1) showed the effect of *T. nilotica* on the viability of A549 and Wi38 normal human cells lines. The cytotoxic effect of *T. nilotica* crude extract was examined by NR assay that exhibited cytotoxic activity against lung cancer cell line (A549) (IC50 = 50.85 µg/ml). The data showed significant reduction in the viability of the cancer cell which was observed in *T. nilotica* treatment. The crude extract of *T. nilotica* did not induce growth inhibitory effect on normal lung fibroblast cell line (Wi38) (IC50 = 6466 µg/ml).

These data revealed that the *T. nilotica* ethyl acetate crude extract did not affect normal cell lines (Wi38). In contrast, it induced cytotoxicity on cancer cell lines (A549) as shown by a more intense decrease in cell viability on human cancer cell lines. So, these results indicated that *T. nilotica* has antiproliferative effect.

Neutral red uptake assay is a quantitative estimation of the number of viable cells in a culture and one of the most used cytotoxicity tests with many biomedical and environmental applications (Repetto *et al*., 2008).

Results of the cytotoxic assay showed that ethyl acetate extract demonstrated higher cytotoxic activity. Among this plant, the ethyl acetate extract of *T. nilotica* leaves exhibited most potent antiproliferative effect on lung tested cancer cell lines. Noteworthy, the extracts of the most effective plant *T. nilotica* did not produce significant cytotoxic effects against the normal cell lines (Wi38). Altogether, the most biologically active plant which showed significant antiangiogenic as well as cytotoxic activities was *T. nilotica*. The present study indicated the cytotoxic potentials of leaves of *T. nilotica* against human lung (A549) cancer cells.

Orabi *et al*. (2010) agreed with those of who evaluated the cytotoxic effect of *T. nilotica* on different human cell lines such as, promyelocytic leukemia and squamous carcinoma and found that reduction in cells viability of all the human cancer cell lines were used, thus, they reported that *T. nilotica* has anticancer activities. In addition, Boulaaba *et al.* (2013) reported antiproliferative effect of the methanolic extract from halophyte *Tamarix* gallica on human colon cancer (Caco-2) cells. The *Tamarix* gallica significantly inhibited the colon cancer cell growth in a dose-dependent manner.

These results were also in partial agreement with Hassan *et al.* (2014) who found that *T. nilotica* had marked cytotoxicity against the human breast cancer (MCF-7) and colon (HCT 116) cancer

cells while being non-cytotoxic to the tested normal cells. In addition, the plants exhibited significant antiproliferative effects.

Cell cycle analysis using flow cytometry assay

After realizing the high inhibition rate of A549 cancer cell viability by *T. nilotica*, it was necessary to confirm that anti-proliferative effect of *T. nilotica* was associated with cell cycle arrest using flow cytometry-based cell cycle distribution. Figure (2a and b) showed that, compared with the control group, *T. nilotica* at 50.85 µg/ml markedly affected the cell cycle distribution in A549 cells. The G0/G1 phase showed reduction from 49% to 37%, while the S-phase was slightly decreased from 24% to 21% in the control and *T. nilotica*, respectively. On the other hand, the percentage of A549 cells at G2/M phase was highly increased after *T. nilotica* treatment to about 1.55 folds (from 27% to 42%) as compared to the control. These results showed significant accumulation of A549 cells in the G2/M phase, and confirmed that *T. nilotica* has cytotoxic effect on cancer cells via induction of G2/M phase arrest of the cell cycle.

Cho *et al*. (2005) reported that the control of cell division is the major regulatory mechanism of cell growth; the cell cycle analysis is a novel and suitable method for cancer control and eradication. There is relationship between cell cycle and apoptosis, and together they play an important role in the sensitivity of cancer cells to chemotherapy, whereas, accumulation of cells in subG1 phase was an indication of apoptosis. (Haruyo *et al.*, 2006).

`Our findings showed that A549 cells were blocked at the G2/M phase following 24 h exposure to *T. nilotica* crude extract at 50.85 μg/mL. This was in partial agreement with a study on colon cancer (Caco-2) cells treated with *Tamarix gallica* extract, which suggested that *T. gallica* could be useful as a candidate source of antiproliferation molecules (Boulaaba *et al*., 2013). The *T. gallica* powerful antioxidant activity is due to containing several phenolic compounds (Ksouri *et al*., 2009).

However, our results were in disagreement with those of Farha *et al.* (2013), who reported that the analysis of cell cycle revealed the induction of apoptosis by accumulating cells in G1 phase and G2/M cell cycle arrest. Anti-cancer compounds arrest the cell cycle at the G1, S, or G2/M phase and then induce apoptotic cell death pathway. Sleiman and Stewart (2000) suggested that many cytotoxic molecules induce mitotic cell death (apoptosis) which occurs in parallel with G2/M arrest.

Analyses of apoptotic cells by (AO/EtBr) immunofluorescence double staining

Apoptotic nature of the cell death caused by crude extract of *T. nilotica* were evaluated through nuclear changes by EtBr/AO double staining technique. Apoptotic activity from *T. nilotica* ethyl acetate crude extract was evaluated with respect to the morphology of cells by fluorescence microscopy. Compared with

spontaneous apoptosis which was observed in negative control cells, A549 treated with 50.85 µg/ml. The crude extract showed an increase of apoptosis. Regarding the control, the nuclear region of viable cells was uniformly green in color, whereas, early apoptotic cells were orange with bright dots corresponding to nuclear chromatin fragmentation. Late apoptotic cells showed intensive red nucleus. Cells treated with *T. nilotica* extract exhibited characteristic changes of apoptosis e.g. fragmentation and formation of apoptotic bodies, as shown in Fig. (3).

These findings were further supported by the results of analyses of apoptotic cells by (AO/EtBr) immunofluorescence double staining. The results showed that, the extract of *T. nilotica* displayed strong (AO/EtBr) quenching ability with lowest IC50 (9 µg/ml).

One of the helpful strategies for anticancer drug development is the induction of apoptosis in cancer cells (Hu and Kavanagh, 2003). Apoptosis plays a significant role in deleting the mutated hyperproliferating cells from the system. Thus, inducers of apoptosis have been utilized in cancer medication and stimulation of apoptosis paths is a key mechanism by which cytotoxic medicines kill tumor cells and are being considered as an important technique for measurement of the clinical efficiency of some anti-cancer drugs (Earnshaw *et al.*, 1999).

The method of Acridine orange/ Ethidium bromide (AO-EB) staining has been mentioned in studies of apoptosis in human lymphoid. This application was based on differential uptake and DNA staining of AO and EB, equilibrium at low concentrations (Liegler *et al.*, 1995). The AO/EB procedure is used to detect apoptosis and can differentiate among early apoptotic, late apoptotic and lifeless cells as OA/EB is a more convenient and economical method (Liu *et al*., 2015).

Acridine orange is a dye that stains both live and lifeless cells; Ethidium bromide only stains dead cells that have missed membrane integrity. Live cells stain uniformly green. In contrast, early apoptotic cells stain green and contain bright green dots in the nuclei. Late apoptotic cells also incorporate EtBr displays condensed and fragmented orange chromatin. On the other hand, EtBr penetrated into the membranes of necrotic cells and stains their nuclei orange color, but present nuclear morphology resembling that of viable cells (Farha *et al.*, 2013).

Our results partialy agreed with those of Monga *et al.* (2013), who used AO/EB immunofluorescence staining to apoptosis cells detection and showed that *Acacia catechu* ethanol extract has antiproliferation effect on HepG2 cells and SCC-25 cells via apoptosis induction (Lakshmi *et al*., 2017).

1.1. Determination of the expression levels of apoptosis-regulatory genes:

The expression levels of some apoptosis-related genes; p53, Bcl-2 and Bax in A549 cells were determined by quantitative real time PCR. Figure (4) showed

that, compared to the control, the expression levels of *p53* and *Bax* were increased, whereas that of *Bcl-2* was decreased.

These results indicated that *T. nilotica* had probably killed A549 cells through apoptosis mechanism mainly *via* the over-expression of *p53* and *Bax* genes, while *Bcl-2* was down-regulated.

The p53 gene was reported to modulate susceptibility of cells to apoptosis by down regulation of Bcl-2 and by causing up-regulation of Bax (Choi *et al*., 2000).

A number of studies focused on the essential role of *p53* in the balance between apoptosis and proliferation (Polager and Ginsberg, 2009). The *p53* gene play a key role in G2 checkpoint, suppresses G2/M transition. In addition,it regulates the balance between the pro-apoptotic *Bax* gene and the anti-apoptotic *Bcl-2* gene through its transcriptional activities (Leu *et al*., 2004). *Bcl-2* genes play a crucial role in controlling the mitochondrial pathway of apoptosis (Dewson and Kluck, 2009), which consists of pro-apoptosis genes (Bax, Puma, Bim, Noxa, Bid), anti-apoptosis genes (*Bcl-xl*, *Bcl-2*), and one of the mitochondrial permeability transition pore (Chen and Lesnefsky, 2011). *Bcl-2* can stabilize the mitochondria permeability transition and avoid the release of cytochrome c to inactivate caspase (Li *et al*., 1997).

(Youle and Strasser, 2008), stated that mRNA expression levels of apoptotic-related genes and proteins, *p53*, *Bax* and *Bcl-2* in HepG2 cells treated with the *A. halimus* extract induces apoptosis which was elicited through *p53*, *Bax* upregulation and down regulation of *Bcl-2* genes. This finding is in agreement with many studies which demonstrated the role of *p53*, *Bax* and *Bcl-2* in inducing apoptosis

In conclusion, these results have established that *T. nilotica* exhibited substantial anticancer activity against lung carcinoma cell lines. The data suggest that these medicinal halophytes might be valuable sources of bioactive secondary metabolites and a promising source of health products for functional food or nutraceutical industries.

SUMMARY

Samples of *Tamarix nilotica* were collected from Wadi Gharandal, South Sinai, Egypt in March 2015 by Desert Research Center (DRC).

Plants was extracted by Ethyl acetate. Possibility of anticancer activity of crude extract was assessed against human lung carcinoma (A549) cell lines to identify the best extraction solvent. Results showed the extract of plant was significantly active against the human lung carcinoma (A549). *Cytotoxic activity onA549 andWi38 cell lines by Neutral red assay indicated that T. nilotica* ethyl acetate crude extract did not affect normal cell lines (Wi38). In contrast, it induced cytotoxicity on cancer cell lines (A549) as shown by a more intense decrease in cell viability on human cancer cell lines. Flow

cytometry-basedcell cycle distribution showed that the percentage of A549 cells arrest at G2/M phase was markedly increased after treatment which confirmed the cytotoxic effect on cancer cells So, these results indicated that *T. nilotica* has antiproliferative effect. Analyses of apoptotic cells by (AO/EtBr) immunofluorescence double staining indicated that the crude extract showed an increase of apoptosis. Regarding the control, the nuclear region of viable cells was uniformly green in color, whereas, early apoptotic cells were orange with bright dots corresponding to nuclear chromatin fragmentation. Late apoptotic cells showed intensive red nucleus. Cells treated with *T. nilotica* extract exhibited characteristic changes of apoptosis e.g. fragmentation and formation of apoptotic bodies,

The expression levels of the apoptosis-related genes *p53*, *Bcl-2* and *BAX* in (A549) were assessed by quantitative real time PCR to determine the ability of *T. nilotica* to induce apoptosis in cancer cells (A549), compared with untreated group. Our results indicated that the expression levels of *p53* and *Bax* were increased, whereas that of *Bcl-2* was decreased. The results suggest that *T. nilotica* could be considered good natural source of anticancer agents.

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Table (1): the sequences of primers used for each gene.

Primer	Sequence
p53	F- 5'-CCCAGGTCCAGATGAAG-3' R- 5'-CAGACGGAAACCGTAGC-3'
Bcl-2	F-5'-GGATGCCTTTGTGGAACTGT-3' R-5'-AGCCTGCAGCTTTGTTTCAT-3'
Bax	F-5'-TTTGCTTCAGGGTTTCATC-3' R-5'-CAGTTGAAGTTGCCGTCAGA-3'.

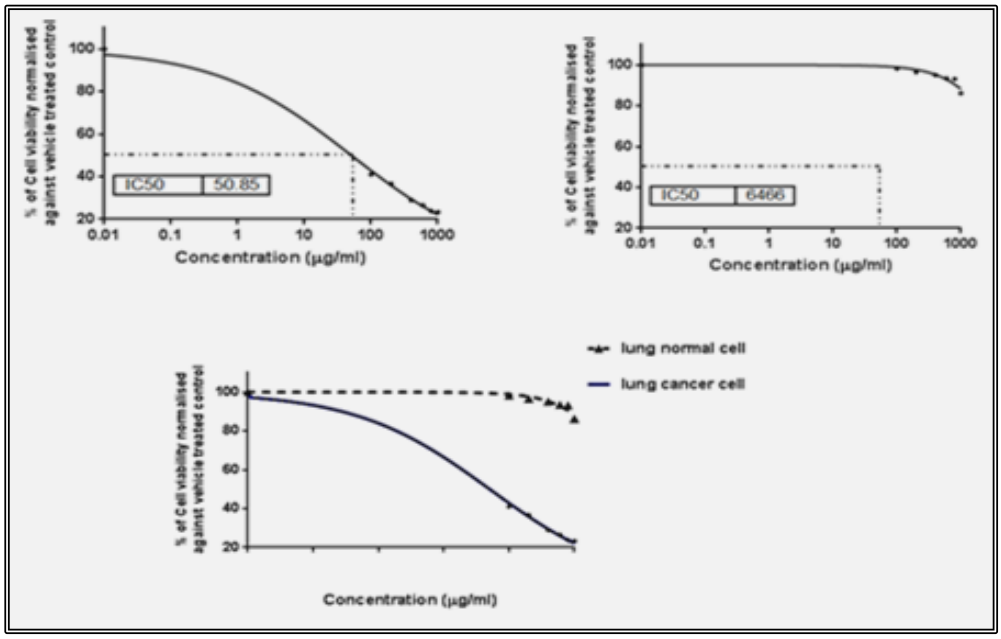
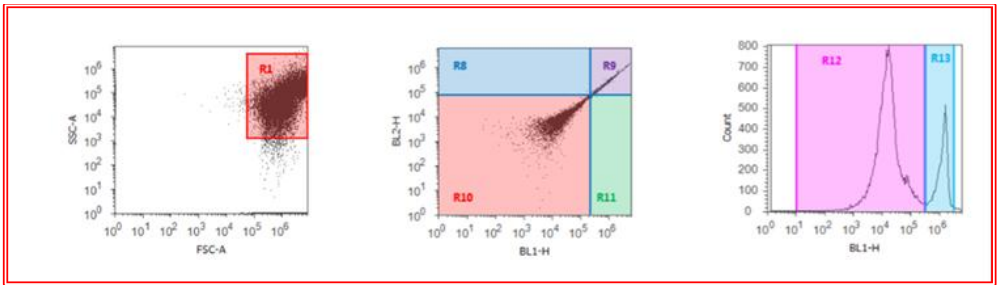
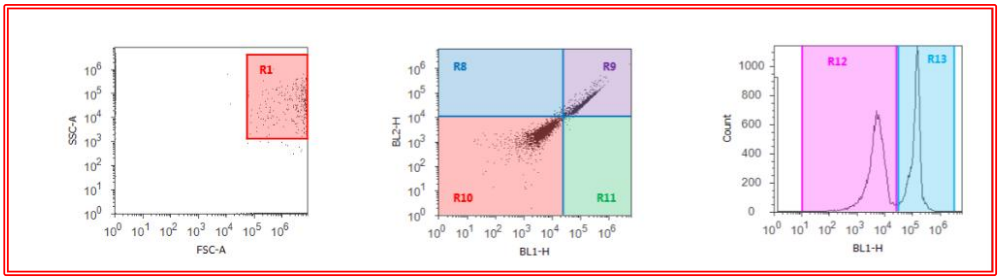


Fig. (1): The IC50 value of the effect of *T. nilotica* on a) human lung cancer cell lines (A549) and b) normal lung cell lines (Wi38).



a) Untreated cells [G0/G1 = 49%, S = 24%, G2/M= 27%]



b) Treated cells [G0/G1 = 37%, S = 21%, G2/M= 42%]

Fig. (2): Effect of *T. nilotica* on the G2/M cell cycle arrest. Lung cancer cells (A549) were treated with *T. nilotica* at the concentration of 50.85 μg/ml in order to check the cell cycle distribution, then analyzed by flow cytometry

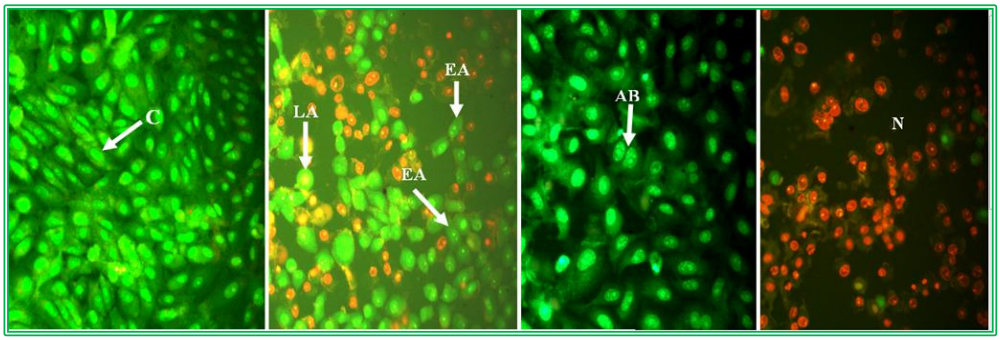


Fig. (3): Effects of Plant ethyl acetate extract on apoptosis induction among lung cancer cells, extract treated by 50.85 µg/mL and stained with AO/EB. The images were taken using fluorescence microscopy at 20×. C: control cells showed green normal nucleus, no treated cells; EA: early apoptosis cells showed green bright nucleus with fragmented chromatin; LA: late apoptosis cells showed intensive red nucleie; AB: apoptotic body; N: necrosis.

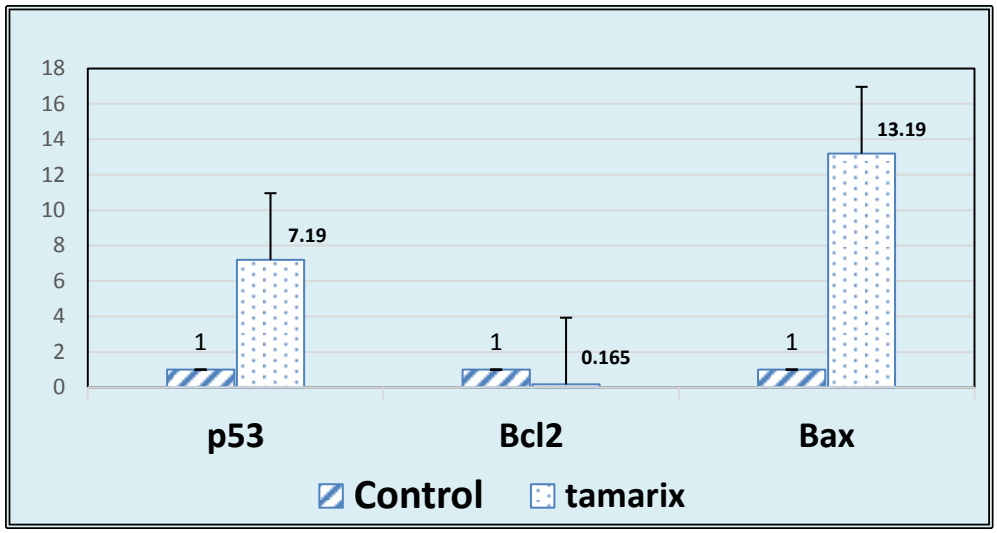


Fig. (4): Effect of *T. nilotica* on apoptosis-related genes after exposure to 50.85 µg/ml, mRNA expression of *P53, Bcl2, and Bax* genes were assessed by quantitative RT-PCR. *P < 0.05, compared to the control group.