Effect of nicotine alkaloid extract of *Nicotiana tabacum* L. on the gene expression of heat shock proteins and apoptosis encoding genes in MCF-7 and Hela cancer cell lines

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**Abstract**---*Nicotiana tabacum* L contains a many active compounds such as Nicotine, which have anti-cancer activities. Apoptosis is a regulated mechanism, and the main feature of it is activating of caspase-9. Also, heat shock proteins overexpression leads to the development of cancer. The current study is aimed to investigate the effect of Nicotine extract at IC$_{50}$ (450, 75) µg/ml on the expression of the (Heat shock protein70, Hypoxia-inducing factor, P53, B-cell lymphoma-2-Associated-X-protein, and Caspase-9) in cancer cell lines, and observation the morphological changes. The quantitative Real-Time Polymerase Chain Reaction is used to analysis of gene expression by using A Kapa syber green stain, and the Acridine Orange:\ Propidium iodide stain also used to observe the morphological changes. The results of this study showed that the treatment of cells with Nicotine extract at IC$_{50}$ inhibits the expression of heat shock proteins encoding genes and increased the expression of apoptosis encoding gene. Also, many morphological changes was observed in apoptotic cells. The study concluded that the extract of Nicotine alkaloid of *Nicotiana tabacum* L. can induce the apoptosis and inhibit the expression of heat shock proteins in cancer cells making this plant is a promising option for cancer treatment, but need more research.

**Keywords**---apoptosis, caspase-9, heat shock protein70, morphological changes, P53.
Introduction

Many countries throughout the world use plants as a key source of medicinal therapy due to their natural healing properties (1). Medical plants are significant in the production of many medicines due to their containing bioactive molecules that offer health benefits such as antibacterial, antioxidant, and anticancer properties (2). The therapeutic benefits of these plants are attributable to the secondary metabolites such as phenolics, terpenoids, flavonoids, and alkaloids which are present in the various portions of these plants (3). *Nicotiana tabacum* L. (Tobacco) is a herbaceous plant, that belongs to the family Solanaceae (4), containing a diversity of active secondary metabolites such as terpenoids, phenols, and alkaloids especially Nicotine, these substances have anti-cancer activities (5). Recent studies have proven that *N. tabacum* L. is a rich source of carotenoids such as β-carotene, which have the anticancer effect derived from its activity in cell cycle arrest (6). The study done by (7) showed that the isolation of cembranoid-type diterpenes (CBDs) from *N. tabacum* L. has a good anti-tumor activity by inhibiting the proliferation of hepatocellular carcinoma cells and altered gene expression, and then induced programmed cell death.

Cancer is defined as the uncontrolled proliferation of cells in the body and is one of the most common diseases in the world (8). It is the second most common cause of death in the world, and the number of people living with cancer reached 21.2 million people in 2021 (9)(10). There are many traditional cancer treatments, such as surgery, chemotherapy, gene therapy, and others, nevertheless failed to produce the desired results and have a long list of side effects (11). According to the FDA, more than 60% of medications used in cancer treatment come from natural sources, due to their effect on the mechanisms of cell division such as DNA replication (12).

Apoptosis is a highly regulated mechanism that generally occurs during the development of cells, and it occurs in cells that are damaged by carcinogenic factors (13). Many features are observed in apoptosis including cells shrinkage, positional organelle loss, damage to mitochondria, chromatin condensation, and DNA fragmentation (14). The initiation of apoptosis is under the control of proapoptotic proteins such as B-cell lymphoma-2-Associated-X-protein (BAX), thus forming a membrane pore that allows cytochrome-C to pass to the cytoplasm (15). Another main feature of apoptosis is activating of Caspases such as caspase-3, -9, and -11 (16).

Heat shock proteins (Hsps) are a large family of molecular chaperones that have roles in protein maturation, and degradation (17). Hsps are classified into many types such as Hsp40, Hsp70, and others (18). Hsps play a key role in a variety of cancer-related activities such as cell proliferation, metastasis, and anti-cancer drug resistance (19). These proteins keep the cells viable under hypoxia, DNA damage response, and many other stress conditions (20). Overexpression of Hsp70 also leads to the development of cancer (21). Also, the Hypoxia-inducing factor (HIF) is overexpressed in many types of cancer, and it has a significant role in promoting malignant tumors (22).
Materials and Methods

Extraction of Nicotine Alkaloid

The extraction of Nicotine alkaloid from *N. tabacum* L. was done as described by (23). Dragendorff’s reagent was used to detect the presence of Nicotine alkaloids in the extract. A brown-orange color that appeared after exposure of the extract to the reagent indicated the presence of Nicotine. The concentrations (50, 100, 200, 400, and 800) µg/ml were used in this study.

Preparation of Cell Lines and Culture

The effect of Nicotine alkaloid extracted from *N. tabacum* L. on (MCF7) human breast cancer and (Hela) human cervical cancer cell lines by in vitro technique was investigated. The Iraqi Center for Medical and Genetic Research’s standard approach was used to prepare the solutions. These cells were cultured for 24 hours in RPMI–1640 media with 10% (v/v) calf fetal serum and 5% CO2.

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

- **Seeding of Cells**
  Two culture containers with a size of 25 cm² were used to implant the cells for each of the MCF7 and Hela cancer cell lines at a rate of one million cells per container. The cells were incubated at 37°C for 24 hours until the adhesion and proliferation of cells happen in each container and the formation of a monolayer.

- **Exposure of Cells**
  After the formation of the monolayer, the cells were exposed to Nicotine alkaloid extract at IC_{50} for each line for 24 hours, and one culture container for each cell line was left untreated as a positive control.

- **Cell Harvesting**
  A sterile scraper was used to harvest cells without removing the culture medium, collected in sterile test tubes, and transferred to a centrifuge for cell sedimentation, then the culture medium was discarded, the precipitate was washed with a solution of Phosphate Buffer Saline (PBS), then the cells were suspended in 50 µl of cooled PBS and kept at -80 °C.

- **Extraction of RNA**
  The abm EXCellenCT Lysis Kit (Abm, Canada) was used to extract RNA from cultivated cells after they were thawed at room temperature according to the manufacturer’s instructions. The First Chain cDNA Synthesis Kit (TonkBio, USA) was used to synthesize cDNA from pure extracted RNA according to the manufacturer’s instructions. A Nano-drop spectrophotometer (Thermo Scientific, U.S.A) was used to evaluate the purity and concentration of cDNA.

- **Gene Expression Analysis by Using Quantitative Real-Time PCR (qRT-PCR)**
  The quantities of mRNA for (P53, BAX, and Caspase-9) and (Hsp70 and HIF) in MCF7 and Hela lines of cancer were measured by using qRT-PCR (Agilent MX3005P, Germany) with the Glyceraldehyde-3-phosphate housekeeping gene (GAPDH) serving as a positive control. The study primers were made by the Iraqi Biotechnology Company and were matched to the National Center
for Biotechnology Information (NCBI) and stored freeze-dry at -20°C. All genes used in this study were designed by using the NCBI Primer-Blast program as shown in (Table 1).

A Kapa syber green master mix kit from (Kapa, USA) was used for the qRT-PCR reaction. The reaction was carried out in a 20 µL volume that included 2 µL of cDNA (100 ng), 10 µL of KAPA SYBR Green Master Mix, 6 µL of RNase-free water, and 1 µL of each primer (Forward and Reverse) at a concentration of 100 µM. (Table 2) shows the thermal profile of qRT-PCR for genes expression used in this study.

<table>
<thead>
<tr>
<th>Step</th>
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<th>Period</th>
<th>Cycle</th>
</tr>
</thead>
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<td>30 second</td>
<td>Hold</td>
</tr>
<tr>
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<td>Elongation/Extension</td>
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Morphological Changes Detection by Using Acridine Orange/Propidium Iodide (AO/PI) Stain

**Principle**

The Acridine orange (AO) and Propidium iodide (PI) kit (US biological, U.S.A) is a double-fluorescence stain. It is a way of assessing apoptotic cell morphology by using nuclear DNA staining. AO is a cationic dye that can stain all nucleated cells and trigger green fluorescence in both live and dead cells, whereas PI can enters only to dead cells with broken membranes and produces red fluorescence.
Procedure

- This method used a 24-well culture plate. 200 µl of the cultured medium that was incubated with CO₂ was added and incubate for 24 hours at 37°C.
- After Discarding the medium, treated the cells with Nicotine alkaloid extract at IC₅₀ and incubated for 24 hours.
- After the end of the incubation period, remove the mixture exposed, and Add 50 µl of the AO/PI mixture to each well of the plate and incubated in the incubator of co₂ for 20-30min in dark at 37°C.
- After that, the stain mixture is removed from the wells and rinsed the wells with PBS, Repeat this step one more time to remove any presence of stain.
- Finally, 50 µl of PBS was added to each well of the plate directly. Microphotographs were taken by using an inverted fluorescent microscope (Oltmpus, Japan) with blue and green filters.

Results

Extraction of RNA from (MCF7 and Hela) Cancer Cell Lines

Total RNA was isolated from MCF7 and Hela cancer cell lines that had been treated for 24 hours with Nicotine alkaloid extract at IC₅₀ (450 and 75) µg/ml, respectively. Using a Nano-Drop Spectrophotometer, the concentration of RNA that was isolated from each cancer cell line that was converted to cDNA was determined, and it ranged from (1288-1312) ng/microliter, with purity between (1.79 and 1.80).

Effect of Nicotine Alkaloid Extract on Heat Shock Protein (Hsp70 and HIF) Gene Expression in Cancer Cell Lines (MCF7 and Hela)

RT-PCR and Syber green dye, a fluorescent dye that can differentiate any DNA, even complementary DNA, were used to measure the degree of gene expression in cancer cell lines. Treatment of cells with Nicotine alkaloid extract at IC₅₀ (450, 75) µg/ml for 24 hours inhibits the gene expression of heat shock proteins expressing genes. In the MCF7 cancer cell line, gene expression of the (Hsp70 and HIF) was (0.42, and 0.14), respectively, in treated cells compared to the control group, while in the Hela cancer cell line, it was (0.10, and 0.76) in treated cells compared to the control group. (Table 3) and (figure 1) shows the changes in gene expression in both cancer cell lines.

Effect of Nicotine Alkaloid Extract on apoptosis (P53, PAX, and Caspase 9) Gene Expression in Cancer Cell Lines (MCF7 and Hela)

The treatment of (MCF7, Hela) cell lines with Nicotine alkaloid extract at IC₅₀ (450 and 75) µg/ml, respectively, for 24 hours increased the expression of the Caspase9 encoding gene. The percentage change in the level of expression of the Caspase9 encoding gene was (1.90 and 2.69), in (MCF7 and Hela), respectively, in cells treated with Nicotine. While the expression of p53 and BAX encoding genes in the MCF7 was 0.13 and 0.47, respectively, whereas, in the Hela, it was 0.17 and 0.53, respectively. (Table 4) and (figure 2) shows the changes in gene expression in both cancer cell lines.
**Observations of Morphological Changes by using Acridine Orange \ Propidium iodide (AO/PI) Stain**

The Acridine Orange and Propidium iodide are a double stain used as a great procedure to detect the apoptosis properties induced in (MCF7 and Hela) cancer cell lines as a result of exposure to the extract of Nicotine alkaloid of *N. tabacum L.* for 24 hours with IC$_{50}$ (450 and 75) µg/ml. Using fluorescent microscopy, the morphology of apoptotic and live cells was distinguished based on the integrity of the cell membrane. According to these findings, 24 hours of incubation with extract of Nicotine alkaloid was sufficient to induce a high proportion of the cell population to the late stages of apoptosis.

After staining the cells with a mixture of these stains, three types of colored cells can be seen under a fluorescing microscope. All nuclei in control live cells turned green (in blue fluorescence) and disappeared (in green fluorescence) as AO penetrated the cell membrane, demonstrating a normal spherical structure and chromatin organization. The nuclei of early apoptotic cells were green-yellow or orange in color (in blue fluorescence) and light in color (in green fluorescence), and they were either condensed or fragmented. Late apoptosis was observed as an orange or reddish-orange colored (in blue fluorescence) and brightly colored (in green fluorescence) cell death with nuclei fragmentation and condensation due to the PI band of DNA cell deaths. (Figure 3,4) describe these morphological changes in MCF7 and Hela cell lines, respectively.

(Table 3): Gene expression change of (Hsp70, HIF) in (MCF7 and Hela) cell lines

<table>
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<th>Hela</th>
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<tbody>
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<td>0.10</td>
</tr>
<tr>
<td>HIF</td>
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<td>0.76</td>
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(Table 4): Gene expression change of (P53, PAX, and Caspase 9) in (MCF7 and Hela) cell lines

<table>
<thead>
<tr>
<th>Genes</th>
<th>MCF7</th>
<th>Hela</th>
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<tbody>
<tr>
<td>P53</td>
<td>0.13</td>
<td>0.17</td>
</tr>
<tr>
<td>PAX</td>
<td>0.47</td>
<td>0.53</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>1.90</td>
<td>2.69</td>
</tr>
</tbody>
</table>
(Figure 1): Gene expression change of (Hsp70, HIF) in (MCF7 and Hela) cell lines

(Figure 2): Gene expression change of (P53, PAX, and Caspase 9) in (MCF7 and Hela) cell lines
(Figure 3): Morphological changes of apoptosis detection by using (AO\PI) stain in MCF7 cells that exposure to extract of Nicotine alkaloid for 24 hours at IC_{50} (100X). a) control cells; b) treated cells: (a1, b1 “fluorescence of blue”), (a2, b2 “fluorescence of green”), and (a3, b3 “visible fluorescence”). Red arrows refer to living cells, while yellow arrows refer to dying cells.
(Figure 4): Morphological changes of apoptosis detection by using (AO\PI) stain in Hela cells that exposure to extract of Nicotine alkaloid for 24 hours at IC$_{50}$ (100X). a) control cells; b) treated cells: (a1, b1 "fluorescence of blue"), (a2, b2 "fluorescence of green"), and (a3, b3 "visible fluorescence"). Red arrows refer to living cells, while yellow arrows refer to dying cells.

**Discussion**

Heat shock proteins have a wide range of functions in the suppression of programmed cell death (24). Hsp70 overexpression has been indicated as a marker in some malignancies (25). Because Hsp70 acts as an anti-apoptotic factor, it has been identified as a possible target for anticancer treatment (26). The chloroform, methanol, and ethyl acetate extracts of the roots of *Hemidesmus indicus*, seeds of *Nigella sativa*, and rhizomes of *Smilax glabra* showed a clear down-regulation of Hsp70 gene expression level in the non-small-cell lung (NCI-H292) cancer cell line (27). Overexpression of HIF-1 in many types of cancers promotes cancer progression through a variety of pathways, such as reprogramming of metabolic processes to compensate for the hypoxia, and then metastasis (28). The hexane and methanol extracts of *Baeckea frutescens* leaves
and branches that belonged to the Myrtaceae family showed a significant effect on the expression of HIF in the MCF7 breast cancer cell line (29).

Apoptosis is an evolutionarily conserved cell death mechanism that is involved in proper eukaryotic development and the maintenance of organismal homeostasis (30). The methanol and acetone extracts of the fruit of *Vatica diospyroides Symington* showed an effective impact in increasing the expression of BAX protein in the Hela cervical cancer cell line. This leads to the release of cytochrome-C from mitochondria and binding with Apaf-1 and then with caspase9 to form a complex called apoptosome and this leads to apoptosis occurring (31). Also, the alkaloid extracted from *Cyperus rotundus L.* by using methanol 80% can affect the gene expression of P53 in human digestive system cancer cell lines (SKGT-4 and HRT), where the treatment of cells with alkaloid for 24 hours of incubation leads to up-regulation of the P53 (32).

Many morphological changes can be observed during apoptosis (33). The ethyl acetate extract of leaves of *Phyla nodiflora L.* has a cytotoxic effect on the MCF7 breast cancer cell line by indicating DNA fragmentation (34). Also, the aqueous and methanol extracts of the whole plant of *Anastatica hierochuntica L.* cause degradation in chromosomal DNA in the MCF7 cell line after 24 hours of exposure to 400 µg/ml of extract (35). The study indicated by (36) shows that the aqueous extract of unfermented *Eurycoma longifolia* leaves shows an inhibitory effect on the MDA-MB-231 and MCF7 breast cancer cell lines through fragmentation of DNA and release of cytochrome-C. Also, a study was done by (37) showed that the treatment of colorectal HT-29 cancer cell line with the alkaloid extract of *Stachys pilifera* has a significant effect on morphological changes such as shrinkage of cells, and cell debris, and forming of apoptotic bodies.

**Conclusion**

The study concluded that the extract of Nicotine alkaloid of *Nicotiana tabacum L.* can induce apoptosis and inhibit the expression of heat shock proteins in cancer cells making this plant is a promising option for cancer treatment, but more research is needed to verify it.

**Acknowledgment**

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**References**


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