

Alkaloid Extract of *Equisetum arvense* Plant Induce Apoptosis in MCF7 Cancer Cell Line.

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Abstract:

This study was conducted to detect the ability of an alkaloid extracted from *Equisetum arvense* to induce apoptosis on MCF7 cancer cells. The IC₅₀ concentration was employed in these experiments (325 µg/ml at 24hr), it was obtained from studying the effect of the alkaloid extract on the growth inhibition of cancer cells after 24 hours of exposure. genes involved in apoptosis (P53, BAX, Caspase8, and Caspase9) were assessed on the mRNA level. besides that, a study for the cells morphological changes that occurred via apoptosis was carried out by staining with a mixture of two fluorescence dyes acridine orange and propidium iodide. Mitochondrial membrane potential was also assessed. The results showed that the expression level of apoptosis genes was elevated. Apoptosis like morphological changes was noticed in cellular membranes and nucleus. Apoptosis characterization of the mitochondria membrane was detected as well. These results revealed the capability of this alkaloid extract to induce apoptosis in this breast cancer cell line. This study confirmed the substantial cytotoxic and pro-apoptotic activity of *Equisetum arvense* alkaloid extract toward the studied cell line, which indicates the utility of this substance in anticancer therapy.

Keywords: *Equisetum arvense*, MCF7 cell line, Apoptosis, Alkaloids.

Introduction:

Humans have suffered since their existence from diseases. However many diseases have been curried with many remedies while cancer remains to be a major health problem human being still facing. Although many cancer medications are in the field of application right now, none of them can bring forth a full recovery without costly side effects (1). Plant metabolites or as it's known as plant secondary metabolites have played an important role in producing different types of cancer therapies. For instance, there are over 60 available chemotherapeutic agents that have been derived from plant agents, as a result, plants can be considered as an essential reservoir of cancer therapies (2). *Equisetum arvense* (*E. arvense*) is traditionally used as medical herbal in oriental countries for healing ulcers, stop bleeding, kidney disorders, and tuberculosis. The plant belongs to the Equisetaceae family and its habitat throughout the northern hemisphere with tempered weather

(3). Considering its long history of traditional use in oriental countries, *E. arvense* meets the requesters for having a medical application with acceptable and proven efficacy (4). The plant was being used in a new approach to studying its cytotoxic effects. Different types of extracts derived from this plant can inhibit cancerous cells' growth with a sort of variation. This variation is rolled by the type of the cell line, the type and procedure of extraction, and on the concentration of the extracted material that incubated with the cells. For the reason of its critical role in tissue homeostasis and cancer development, apoptosis remains to be the most substantial biological process. The modulation of apoptosis either through enhancement or suppression has become an interesting target in the therapeutic strategies of cancer (5). Notable anti-proliferative activity on NIH3T3 mouse fibroblast cell line was seen with ethyl acetate extract of this plant, the cytotoxic action is believed to be in a dose-dependent manner (6). The majority of anticancer drugs used at this time in clinical settings are described to induce cell death by apoptosis (7). In previous research, the ability of *E. arvense* alkaloid extract to inhibit the proliferation of the MCF7 cell line was approved (9). In this research the alkaloid extract of *Equisetum arvense* was used to detect its capacity to induce

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apoptosis event on breast cancer MCF7 cell line.

Materials and Methods:

Preparation of plant material and extraction

The hall plant was gathered from Diyala river area. The plant was identified by botanist Dr. Nidal Idris Sulaiman as *Equisetum arvense* at the Education College/ Department of Biology/ Baghdad University. Alkaloids were extracted as described by Cannell (1998) and Tawfeeq, et. al. (2019) (8 and 9), Dragendorff's reagent was used to detect the presence of alkaloids, after the exposure of the extract to the Dragendorff's reagent an appearance of brown-orange color indicated the presence of alkaloids.

Preparation of MCF7 cell line

Breast cancer cell line MCF7 was cultured in Roswell park memorial institute medium (RPMI-1640) with 10% fetal bovine serum with 1% of ampicillin, gentamicin, and amphotericin. Solutions were prepared according to the standard cell line cultivation method adopted by the Iraqi center of medical and genetic researches. These cells were maintained in the same media and incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

Real-Time Polymerase Chain Reaction (qRT-PCR)

Cell seeding, exposure, and harvesting

Three sets of two 25 mL tissue culture vessels were seeded with MCF7 cell line with 1×10⁶ cells/vessel and incubated in 5% CO₂ incubator at 37°C and 96% humidity for 24 hours to allow cells attachment. After that, One vessel was exposing to alkaloid extract in IC₅₀ concentration (325 µg/ml) (8) for 24 hours and other falcon capped as control (treated with serum-free medium only). After incubation time ended, the cells were scraped and the exposure medium was removed via centrifugation. Harvested cells were washed with 1 mL of BPS and were collected in 1.5 mL Eppendorf tube.

RNA Extraction

RNA was extracted from the cells using abm EXCellenCT Lysis Kit according to the manufacturer instructions (Applied Biological Materials Inc. (abm) 1-3671 Viking Way, Richmond, BC, V6V 2J5, Canada). Complementary DNA (cDNA) was synthesized using the Purified RNA by First Chain cDNA Synthesis Kit (TonkBio, New Road, NJ07054, the U.S.A) according to the manufacturer instructions. The concentration and purity of cDNA were checked with a Q5000 Nano-drop spectrophotometer (Colwell, San Jose, CA 95161 USA).

Quantitative Real time-PCR analysis of genes expression

The mRNA levels were determined by real-time PCR. Primers for P53, BAX, Caspase8, Caspase9, and GAPDH genes were designed using BLAST NCBI (National Center for Biotechnology Information) and their specificity was assessed using UCSC in-silico PCR and the single peak formation during dissociation curve step of the RT-PCR (Table 1). The designed primers were synthesized by IDT (Integrated DNA Technologies, Inc. 1710 Commercial Park Coralville, Iowa 52241, USA.). The PCR amplification reaction was carried out using the KAPA SYBRgreen master mix kit (KAPA Biosystems, Boston, U.S.A). The reaction was performed in a 20 µl volume. Each 20 µl of the qRT-PCR reactions contained 2 µl cDNA (100 ng), 10 µl master mix, 6µl RNase free water and 1 µl of each primer (Forward and Reverse) in the concentration of 100nM using the following thermal profile, enzyme activation 95°C for 30 sec, denature 95°C for 5 sec, and anneal/extend 62°C for 20 sec using 40 cycles. Dissociation thermal profile consist of 95°C for 1 min, 55 °C for 1 min, and 95°C for 1 min with one cycle. The Delta Delta Ct method was used to determine the mRNA level in treated and untreated cells normalized to GAPDH as a housekeeping gene (10). The Agilent Stratagene-Mx3005P Real-Time PCR Detection System (Agilent Technologies, Santa Clara, CA 95051, USA.) was used to perform the reactions. Each gene was detected three times separately with three replicates each time.

Table 1: Real time PCR primers sequences.

| Gene | Primer F/R Sequence 5' to 3' |
|-----------|--|
| P53 | Forward: CCG TCC CAA GCA ATG GAT G Reverse: GAA GAT GAC AGG GGC CAG |
| BAX | Forward: CCT CTC CCC ATC TTC AGA TCA Reverse: TCA AGT CAA GGT CAC AGT GAG |
| Caspase 8 | Forward: GAC CAC GAC CTT TGA AGA GCT Reverse: CAG CCT CAT CCG GGA TAT ATC |
| Caspase 9 | Forward: CTC TTG AGC AGT GGC TGG TC Reverse: GCT GAT CTA TGA GCG ATA CT |
| GAPDH | Forward: GGG TTC TTT GTG CTG AGC GG Reverse: TGC AGA TAG GAA GGG CTT TG |

Acridine Orange/Propidium Iodide (AO/PI) Staining

The cells were seeded in 24 well tissue culture plates at a count of 1×10^5 cells per well. After 24 hr incubation at 37 C in 5% CO₂ and 96% humidity, the cultivated plates were divided into two groups, the first one was treated alkaloid extract in a concentration of 325 $\mu\text{g/mL}$, the second treated with RPMI-1640 serum-free media and served as control. plates were incubate at 37 C in 5% CO₂ and 95% humidity for 24 hours. After incubation was ended, cells were washed with PBS and the washed cells were stained with a mix 1/1 of acridine orange and Propidium iodide fluorescence stains (AO/PI) at concentrations of 4 mg/mL . Stained cells were observed under an inverted fluorescent microscope (Leica DM4000, Leica Microsystems, Germany) and images were taken using CCD camera (Leica MF500, Leica Microsystems, Germany) instilled upon the microscope. Bright field, blue, and red filters were used during cell imaging.

Mitochondrial transmembrane potential assay

The cells were cultivated exactly as described in the previous section and cells exposed to 325 $\mu\text{g/mL}$ of the alkaloid extract for 24 hr incubation at 37 C in 5% CO₂ and 95% humidity. After that cells were washed with PBS and the mitochondrial

transmembrane potential disruption in the cells was detected using Apoptosis Detection, Mitochondria BioAssay™ Kit (United States Biological, 4 Technology Way, Salem, MA 01970. The USA) according to the manufacturer's instructions. Cells were observed under an inverted fluorescent microscope (Leica DM4000, Leica Microsystems, Germany) and images were taken using CCD camera (Leica MF500, Leica Microsystems, Germany) instilled upon the microscope using the blue filter.

Statistical analysis

One way analysis of variance was carried out to determine significant difference using the standard method.

Results:

Quantitative Real-Time PCR

Treatment of the cell line under investigation with the IC50 alkaloid extraction of the *Equisetum arvense* plant (9) induced an increment in the fold of mRNA level of the apoptosis-related gene. The fold of gene expression of P53, BAX, Caspase8, and Caspase9 in treated cells was above that of untreated cells by 0.10, 0.27, 0.76, and 2.57 fold respectively (Figure 1).

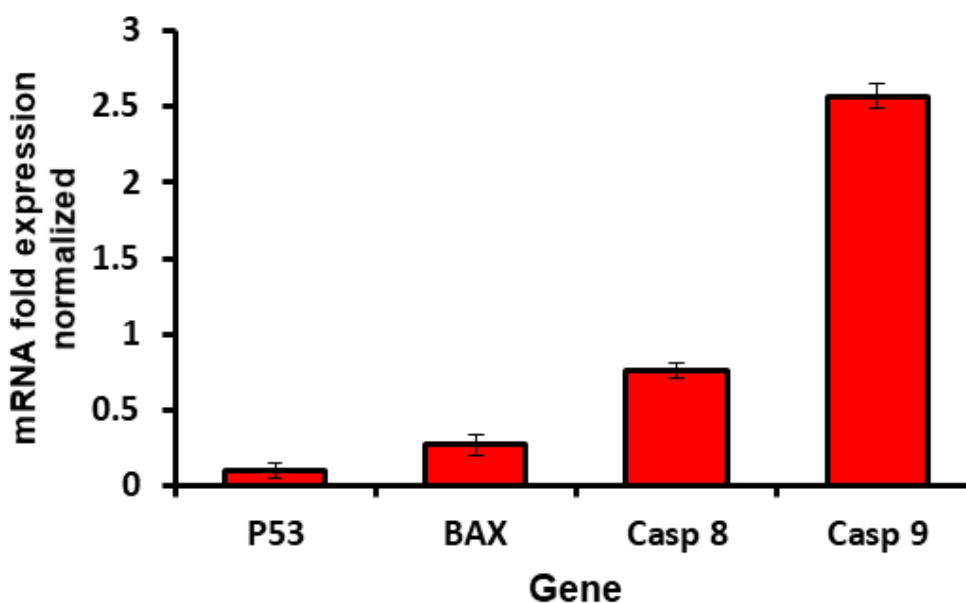


Figure (1): Fold expression increment of apoptosis genes in MCF7 cell line treated with alkaloid extract of *Equisetum arvense*.

Cellular Morphological Changes

The morphological changes in cells treated with the alkaloid extract were enormous (Figure 2). Treated cells suffered from obvious nuclear condensation, cell membrane blebbing, nuclear fragmentation, and cell membrane disintegration. All of these are signs of different stages of apoptosis. As have been seen under bright field and blue/red fluorescence that emits a green/red colors. Dual staining with Acridine Orange/

PI was used to evaluate the nuclear morphology of apoptotic cells. Accordingly, The accumulation of highly intense red fluorescence color in the nucleus of the treated cells indicated a cellular chromatin condensation and that would be followed by nuclear disintegration or fragmentation as can already be seen in some cells. Staining cells cytoplasm with red color indicates that cells are dead or they are on their way to.

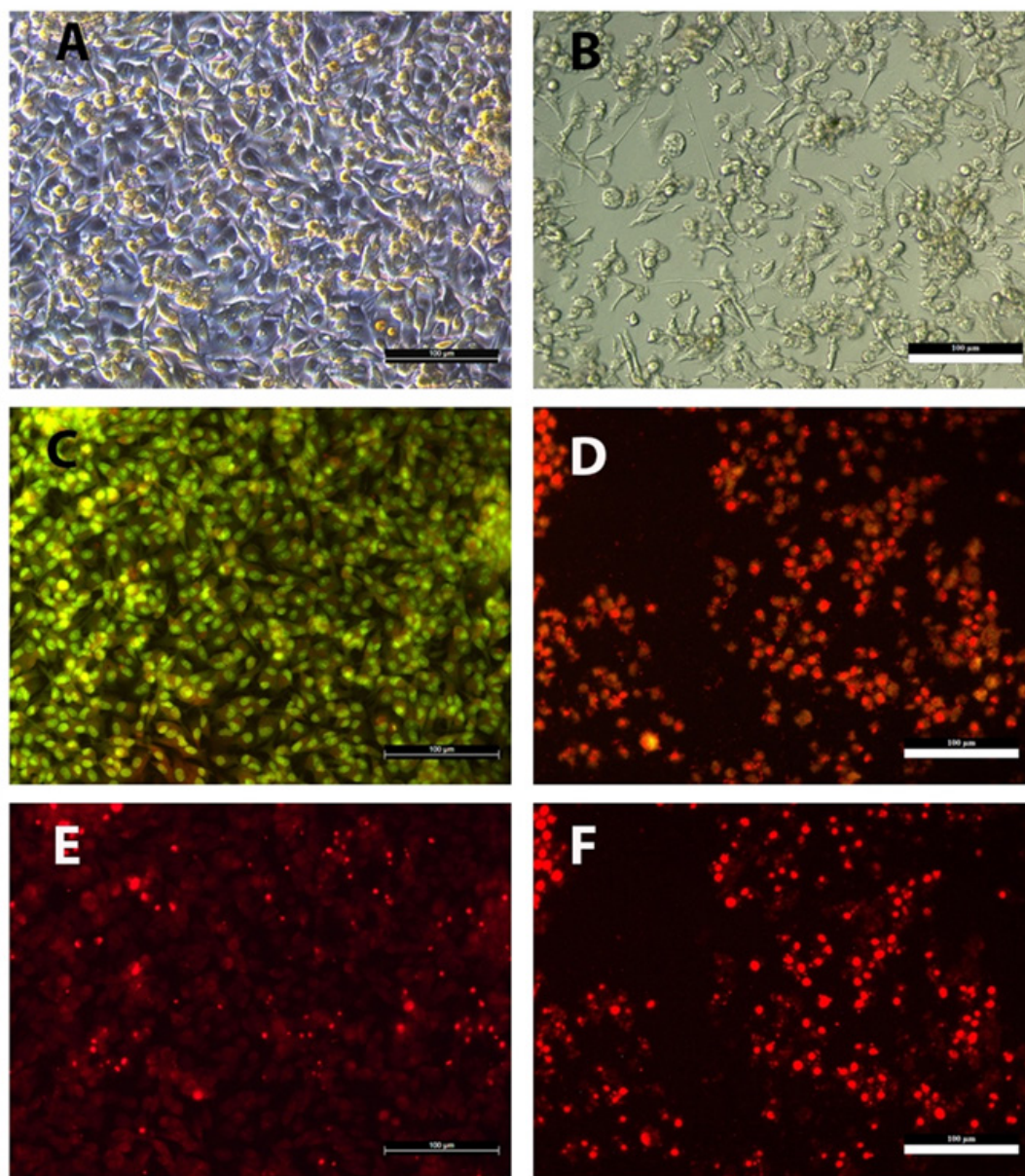


Figure (2): Microphotographs with a fluorescent microscope for MCF7 cells stained with Acridine Orange/Propidium Iodide mixture to observe the cellular morphological changes that indicate different stages of apoptosis. A and B Control and Treatment (in bright field); C, D in blue fluorescence and E, F in green fluorescence.

While staining the cell's nucleus and cytoplasm with green color indicates viable live cells (Figure 2). In general overall, the treated cells expressed an obvious characteristic of apoptosis as a result of the treatment with this alkaloid.

Mitochondrial transmembrane potential

one of the early events of apoptosis is when cells lose their mitochondrial membrane integrity. The exposure of MCF7 cells to the alkaloid extract of the *Equisetum arvense* plant induces that phenomena (Figure 3). The kit used to detect apoptosis contain a cationic fluorescence dye that aggregates

and accumulates at the mitochondrial membrane. As a result of that accumulation the mitochondria will appear to have intensive red dots in them and the cell will appear as if it stained red. However, when the mitochondria lose its membrane integrity the cationic fluorescence stain won't accumulate in their membrane therefore there will be no red color dots in the cells when observed under a fluorescence microscope and the cells will be stained green. Observing the cells after treatment clearly show this case, while the untreated cells look in red fluorescence the treated cells give up a green fluorescence.

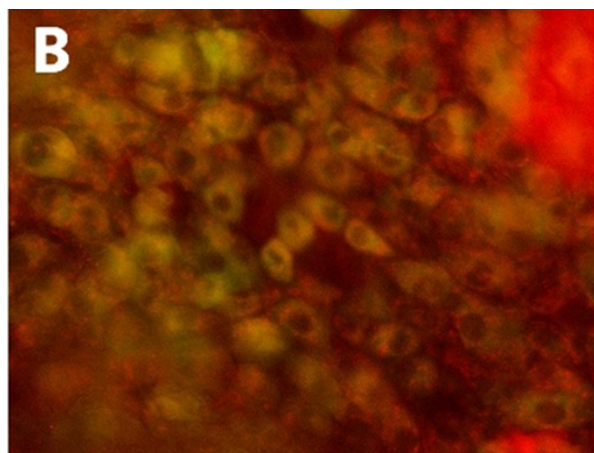
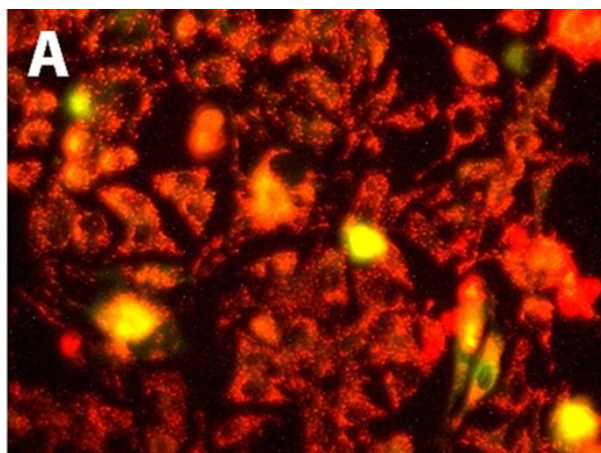


Figure (3): Microphotographs with a fluorescent microscope; Detection of Apoptosis via mitochondrial membrane staining in MCF7 cells. A, Control and B, treated.

Discussion:

Medicinal plants were used for a long time to treat different types of illnesses. People have understood the healing role of these plants since early history. In many oriental countries, herbal medicinal plants are used proficiently in complementary therapies to treat many diseases. Based on hundreds of years of experience with this kind of plant, modern medicine set forth to purify, characterize, a test of activity of many types of a naturally existing chemical compound as active therapies against many maladies and cancer was no exception (11). Unfortunately, despite the significant efforts and billions of money budgets, cancer is still an incurable disease. Therefore, the attempts to find a cure for cancer is continuing. This current study comes following these efforts. Among vital characteristics of any potential therapy for cancer, two of these characteristics by far are the most significant, its capability to induce apoptosis in cancer cells, and its minimal impact against normal cells.

During the last two decades, several studies have shown that several different plant secondary metabolites are competent in provoking apoptosis with a substantial level of toxicity and growth inhibition on cancer cells. Besides that, these studies have concluded that such naturally existing compounds can prevent the initiation of cancer in the first place via well-studied mechanisms. However, some of these compounds faced a profound drawback which is the low level of bio-viability (12, 14). In a previous study, our team has approved that the alkaloid extract of *Equisetum arvense* was a profound growth inhibitor against MCF7 cells with an IC50 concentration of 325 µg/ml as well as having notable antioxidant activity compared with ascorbic acid. Also, This extract did not interfere with normal lymphocyte growth (8, 19). In this study, we go further with the same extract to explore its

potential to induce apoptosis in MCF7 cells. We have used three different techniques to assess that capacity. The level of mRNA for the main apoptosis genes was elevated after 24hr incubation, this represents a piece of substantial evidence. Furthermore, the morphological changes taken place during exposure time showed exactly that of apoptosis, mainly cell membrane blebbing and/or disintegration with clear nuclear condensation and fragmentation. The cells were at different timing of the apoptosis event. Some cells were in the early event, which was characterized when the cells were stained with acridine orange (green) but not with propidium iodide and have obvious nuclear condensation. Other cells were stained with propidium iodide as an indication of death with profound nuclear fragmentation, and that was the main character of late-stage apoptosis. Apoptosis represents a crucial event in the biological processes, it's considered as organized or regulated cell death, therefore it's always preferred to induce during cell life's that suffer from errors during metabolic activities. Reasons behind those desirable cellular events mainly extended to the ability of the immune system to clarify the cellular debris. This matter is highly significant in the case of cancer (14). Two major pathways lead to apoptosis, The death receptor (extrinsic) pathway, and the mitochondrial (intrinsic) pathway. The mitochondrial pathway involved the induction of specialized protein that induces mitochondrial leakiness, leading to the release of cytochrome C which binds to a protein called apoptosis-activating factor-1 (APaf-1) a death-inducing protein (15). The alkaloid extract used in this study was capable to change the mitochondrial membrane potentiality, this was evident in the third technique that has been invested in this study. Cells treated with the IC50 concentration for 24hr have their mitochondria damaged and that damage defiantly induces apoptosis. Therefore this alkaloid induces apoptosis via the intrinsic pathway. This

was supported by the results obtained from the real-time PCR experiments where the Bax mRNA was elevated too. Other studies that used medicinal herbal extracts have shown similar results. The aqueous extract of *Vetiveria zizanioides* roots demonstrated a profound cytotoxicity towards MCF-7 cells via stimulating the fragmentation of nuclear material and the intensification of chromatin (16). While Berberine has an inhibitory effect on HL-60 leukemia cells because of its ability to condense chromatin and the fraction of nuclear material (17). It was regarded that the alkaloid extract of *Peganum harmala* has an inhibitory effect on cancer cells by stimulating the fragmentation of nuclear material with the release of

cytochrome C cells (18). All these studies confirm the influence of plant extracts on the induction of apoptosis through their impact on many cellular activities, and some of which were detected in the current study. In conclusion, the alkaloid extract of *Equisetum arvense* was used to detect its apoptosis effect on the MCF7 cancer cell line, alkaloid extract was induced apoptosis by both the intrinsic mitochondrial pathway and extrinsic after 24 hours of exposure. According to the acridine orange/propidium iodide mixed fluorescent staining assay, the used alkaloid extract was able to induce apoptosis in the treated cells after 24 hours of exposure.

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