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The effect of aqueous and ethanolic extracts of *Artemisia herba alba* on human laryngeal carcinoma and murine mammary adenocarcinoma cell lines

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Abstract

The present study was carried out to evaluate the cytological effects of aqueous (AE) and ethanolic (EE) extracts of *Artemisia herba alba* on human laryngeal carcinoma (Hep-2) cell line and murine mammary adenocarcinoma (AMN-3) cell line *in vitro***.** The cytological study performed simultaneously with cell growth assay. The results of study revealed concentration-dependent cytological changes like patchy growth inhibition, loss of confluent feature and cellular degeneration after exposure to the lowest concentrations (156.25 and 312.5 µg/ml). The early findings of cytolysis were seen after exposure to 625 µg/ml. While the highest concentrations (1250, 2500 and 5000 µg/ml) caused severe growth inhibition with marked cytolytic features including loss of cellular outlines, large numbers of dead cells and high content of cellular debris. In conclusion, the results of this study revealed the high cytological effect of *Artemisia herba alba* extracts on Hep-2 and AMN-3 cell lines *in vitro*.

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Introduction

Herbal medicines are culturally accepted and widely used in many countries for treatment of disorders and hence are of great importance as a mechanism to increase access to health care services. However, only few countries have some forms of policy/mechanism on traditional/complementary and alternative medicine (TCAM). Other countries need to

develop their policy on TCAM to provide a sound basis in defining the role of TCAM in national health care delivery, ensuring that necessary, regulatory and legal mechanisms are created for promoting and maintaining good practices, that access is equitable, affordable and that authenticity, safety and efficacy of therapies are ensured (El- Gendy, 2004). Recently synthesized drugs started to replace natural ones due to many well-known reasons. But after the increase of drug industry and modern technology, man began to test plant products due to some harmful side effects or symptoms caused by some synthesized drugs (Oran and Al-Eisawi, 1998). *Artemisia herba-alba* (*A. herba-alba*) is extensively used in Iraqi folk medicine. It is a popular medicinal plant that has been used in the Middle East classical medicine for treating diverse diseases. It is used by local people of some countries as an anti-diabetic (Kamal *et al.,* 2007; Ashraf *et al.,* 2010). *A. herbaalba* mixtures f have been used as analgesic, antibacterial, and hemostatic agents (Tilaoui *et al*., 2011; Mohamed *et al.,* 2010). It is used in Jordon in the form of a decoction against fever, menstrual and nervous problems (Abad *et al*., 2007). The essential oil of this herb was found to be responsible for its therapeutic use as disinfectant, anthelmintic and antispasmodic (Mohamed *et al.,* 2010). Review of literatures revealed that there were very few scientific and medical studies carried out to assess the effects of *A. herba alba* extract on the cell culture. So, this study intends to investigate the cytological effect of aqueous and ethanolic extracts of *Artemisia herba alba* on the growth of several cancer cell lines *in vitro*.

Materials and Methods

Plant collection

Artemisia herba alba was collected from Al-Najaf province, south of Baghdad in December 2003, and was shed and dried at room temperature. A voucher specimen of the plant was deposited to be identified and authenticated at the National Herbarium of Iraq Botany Directorate in Abu-Ghraib (Certificate no. 3522 in 23/12/2003). The dried plant then was separated into: roots and aerial parts, then the aerial (leaves and phloem) parts were ground into powder by grinder (mesh no.50), and the powdered parts were kept in a plastic bags in deep freeze (-20˚C) until the time of use (Harborne *et al*., 1975).

Preparation of aqueous and ethanolic extracts of *A. herba alba*

According to Harborne *et al*., (1975), aqueous extract of plant was prepared as follows:
1-aliquots of 50 g of the powdered plant were suspended in 200 ml of distilled water (D.W.) in Erlenmeyer flask and stirred on a magnetic stirrer over night at 45˚C.
2-After 24 hours, the sediments were filtered by gauze and then by filter papers.
3-Steps (1) and (2) were repeated 4-5 times.
4-The pooled extract was evaporated to dryness (45˚C) under reduced pressure in rotary evaporator.
5-The weight of crude extract resulted from that amount of powdered plant was measured.
6-The crude extract then was kept at -20˚C until the time of use.

7-The ethanolic extract of *A. herba alba* was prepared in the same manner as that of the aqueous extract except using of 70% ethyl alcohol instead of D.W. For following experiments, 1 g of powdered plant extract was dissolved into 100 ml PBS (as solvent), the suspension then filtered and sterilized by using 0.4 μm sterile millipore filter and kept in deep freeze (-20˚C) until use.

Figure. 1: Shows *Artemisia herba alba*

Cytological Study

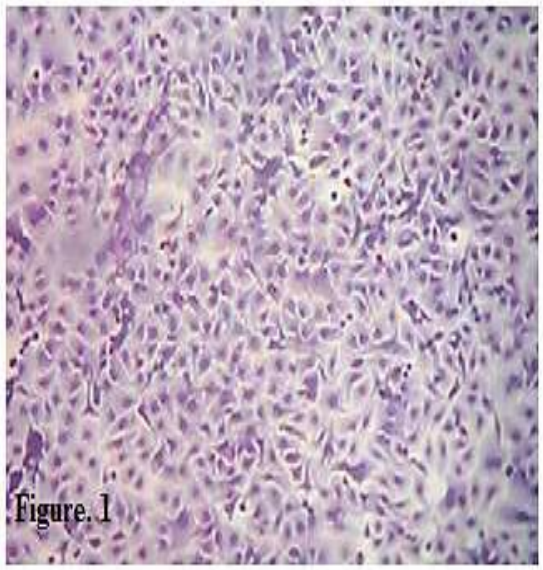
The aqueous and ethanolic extracts prepared from *Artemisia herba alba* were tested on human laryngeal carcinoma (Hep-2) cell line and murine mammary adenocarcinoma (AMN-3) cell line. The cells were cultured with different concentration of plant extracts 156.25, 312.5, 625, 1250, 2500 and 5000 μg/ml and cultured for 72 hrs. Later on, these cell lines were fixed after in 10% PBS/ formalin solution and stained with hematoxylin and eosin to study the morphological changes of the tested cells (Luna, 1968).

Results

Cytological study

In the present study, both types of cell lines were examined microscopically after 72 hrs of exposure to the aqueous extract. The results revealed marked cytological changes. These changes were diverse depending on the concentration of the extract. The pattern of growth inhibition seems to be similar for both AMN-3 and Hep-2 cell lines. The Hep-2 cell line of the control group showed complete confluent monolayer of cohesive

malignant cells (Figure. 1), which were pleomorphic, hyperchromatic,well differentiated with high nucleus/cytoplasm ratio. The cells contain prominent nucleoli, even some cells have more than one nucleoli (Figure. 2). Formation of multinucleated giant cells could be seen (Figure. 3). Some of these monolayers showed an evidence of overlapping of small dark proliferating cells.



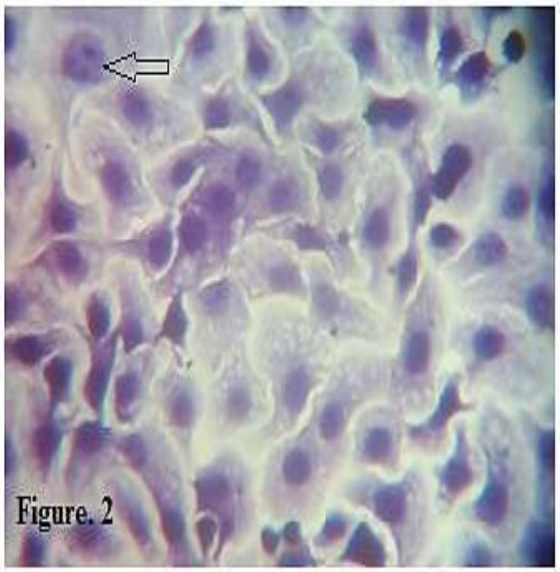


Figure.1: Hep-2 cell line shows confluent monolayer, no Empty spaces, cohesive malignant cells (control group). (100X, H&E stain).
Figure.2: The control group of Hep-2 cell line reveals cohesive, pleomorphic, hyperchromatic, well differentiated cells, high nucleus/ Cytoplasm ratio with prominent nucleoli (⇦) (400X, H&E stain).

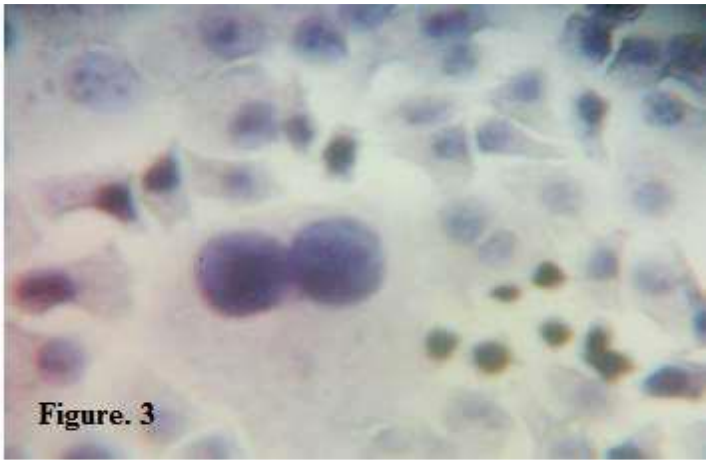


Figure.3: Formation of multinucleated giant cells in the control group of Hep 2 cell line (400X, H&E stain)

At low concentration (156.25 µg/ml) of AE, the monolayer cell growth demonstrated low degree of growth inhibition. At such concentration, the outline cellular features haven't been lost, yet disruption of the monolayer with loss of confluent feature,distinct patchy inhibition with mild cellular swelling and vacuolated cytoplasm as well as number of pyknotic cells were observed (Figure. 4).

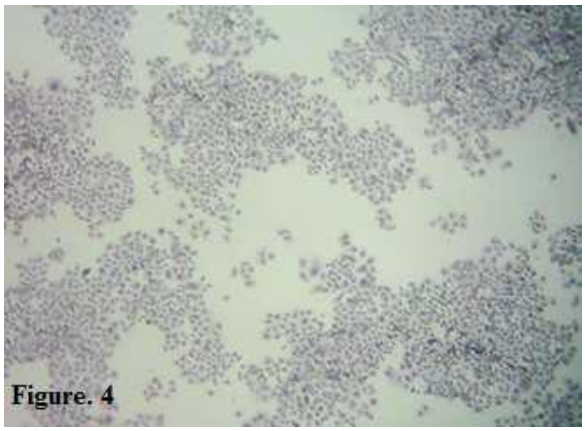


Figure. 4: The monolayer of Hep-2 cell line demonstrates loss of confluent feature, distinct patchy inhibition after exposure to 156.25 µg/ml of AE. (100X,H&E stain).

At higher magnification, condensed nuclear chromatin as a first finding of mitosis (Figure. 5), with characteristic dead cells were detected (Figure. 6). At a higher concentration of AE (312.5 and 625 µg/ml); similar changes with more extensive patchy cellular growth inhibition and earlier stages of cytolysis were noticed. The dead cells became more prominent than that seen in low concentration.

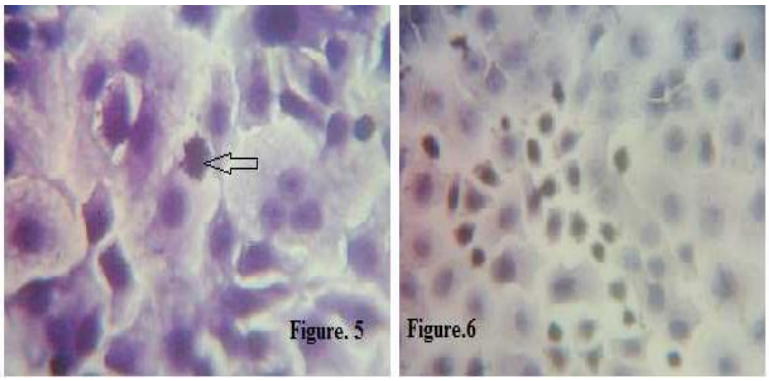


Figure. 5: The cells of Hep-2 cell line shows condensed nuclear chromatin (⇦), cellular swelling and cytoplasmic vacuolation after their exposure to 156.25 µg/ml of AE. (400X, H&E stain).
Figure. 6: Numbers of pyknotic dead cells appeared in Hep-2 cell line after exposure to 156.25 µg/ml of AE. (400X, H&E stain).

On the other hand, the effects of AE appeared to be more severe and the morphological features regarding total lysis of cells with bare nuclei were observed after treatment with highest concentrations (1250, 2500 and 5000 µg/ml). Few cells were retaining their normal features and the examination revealed only the presence of large numbers of dead cells and pyknosis with cellular debris (Figure.7). Depending on the daily microscopic examination of cell lines, it was obvious that there was no important difference in morphological changes between both cell lines of the study. The control group of AMN-3 cell line shared the common characteristic features with Hep-2 cell line, in which there was cohesion of cells with formation of monolayer, no empty spaces were seen and there was an area of overgrowth which appeared darker than usual (Figure. 8). The microscopic changes of AMN-3 cell line after exposure to AE were similar to Hep-2 cell line in both morphology and severity in a concentration- dependent manner (Figures 9, 10, 11).

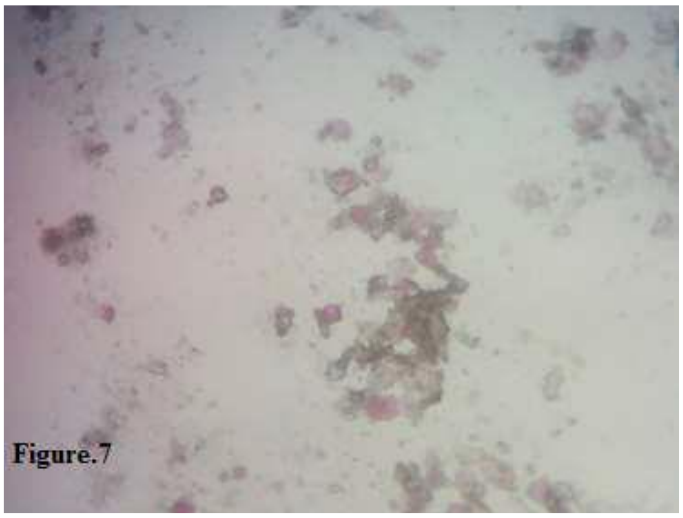


Figure. 7: Hep-2 cell line shows total lysis of cells with cellular debris after treatment with highest concentrations of AE (1250, 2500, 5000 µg/ml). (100X, H&E stain).

Discussion

The most characteristic event recognized in the cytological study was the occurrence of cellular degeneration, cell death and cytolysis in a dose-dependent manner. The cytological findings of Hep-2 and AMN-3 cell line were consistent with that obtained by Hu *et al.*, (2000). They investigated the effect of water soluble macromolecular components of *Artemisia capillaris Thunberg* on hepatoma cell line (SMMC-7721). The effect of these components exhibit morphological changes typical of apoptosis, including condensed chromatin and a reduction in volume. This was consistent with the results of Sa'eed (2004), who tested the effect of green and black tea extract on Hep-2, AMN-3 and fibroblast cell lines. Sa'eed, (2004) was reported the cytological changes similar to those detected in this study. The most accepted explanation for the cytotoxic effect of plant extract is the ability of plants to induce the programmed cell death in cancerous

cells, as attempt to arrest their proliferation. A number of food items as well as herbal medicine have been reported to produce toxic effects by inducing programmed cell death (Thatte *et al.*, 2000). Ferguson *et al.*, (2004) have been isolated an acidified methanol eluate (fraction 6) containing flavonoids and demonstrated anti-proliferative activity. This component was shown in MDA-MB-435 human breast cancer cells to block cell cycle progression and induce cells to undergo apoptosis in a dose-dependent manner.

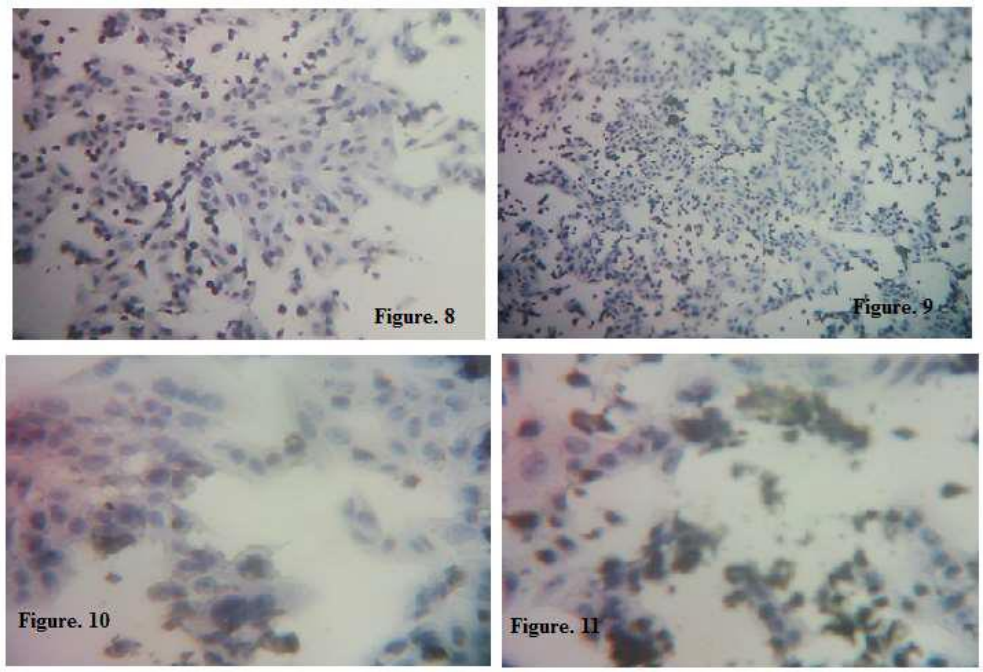


Figure. 8: The cells of AMN-3 cell line characterized by pleomorphism, hyperchromatism, and well differentiation. (200X,H&E stain).
Figure. 9: Patchy growth inhibition and disruption of confluent monolayer after exposure to 156.25 µg/ml of AE. (100X, H&E stain).
Figure. 10: AMN-3 cell line shows evidence of cellular degeneration with presence of large number of dead cells after exposure to 312.5 µg/ml of AE.
(400X,H&E stain).
Figure. 11: AMN-3 cell line reveals great loss of cellular features, large number of dead cells and high content of cellular debris after exposure to high concentrations of AE. (400X, H&E stain).

Several mechanisms have been identified to underlie the modulation of programmed cell death by plants including endonuclease activation, involvement of p53, activation of caspase -3 protease via a Bcl-2 insensitive pathway, potentiate free radical formation and accumulation of sphinganine (Thatte *et al*., 2000). In tissue culture cell lines, the cytotoxic drugs induce the molecular regulator of physiological apoptosis (Dive *et al*., 1992). The cytotoxic drugs induced apoptosis in leukemia and carcinoma cell lines critically depends on activation of caspases (Chen *et al.*, 1996; Los *et al.*, 1997). Caspases are activated by death receptor signaling or are a consequence of mitochondrial

alterations including the release of apoptotic signaling molecules (Sun *et al.*,1999). Another suspected mechanism for plant anti-proliferative action on MCF-7
human breast cell line is induction of an increase of estrogen receptor alpha mRNA. The accumulation of estrogen receptor alpha mRNA (a marker of neoplastic status) was analysed by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (Lambertini *et al*., 2004).
In conclusion, this study approved that *Artemisia herba alba* aqueous extract revealed different cytological effects on the Hep-2 and AMN-3 cell lines *in vitro*. The effects depended on the concentration, the more severe effect increased with high concentration.

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