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Ficus carica Extract Induces Apoptosis through the Mitochondria-Mediated Apoptotic Pathway in Skin Cancer (A2058 Cell Line)

Hasan Jasim Hami ✉
Biology department, College of Science, University of Thi Qar, Thi Qar, Iraq

Roaa J. Elkheralla
Biology department, College of Science, University of Thi Qar, Thi Qar, Iraq

Amal F. Al-Gorani
Biology department, College of Science, University of Thi Qar, Thi Qar, Iraq

Ayam Al Anisi
Education Directorate of Thi-Qar, Ministry of Education, Iraq

Fatima F. Hasheem
Department of Pathological Analysis, College of Science, University of Thi Qar, Thi Qar, Iraq

Abstract

Melanoma is a type of skin cancer that has significantly grown over the last decades. Due to the conventional treatments used for skin melanoma, scientists nowadays pay significant attention to the use of bioactive natural products of medicinal herb. This study's objective was to assess how an alcoholic extract of *Ficus carica* leaves affected on skin cancer cells, apoptosis is triggered (A2058 cell line). In this purpose the anticancer properties of different concentrations (150, 250,350, 450, 550, 650, 750 and 850 µg/ml) of extracts were tested using MTT assay. Meanwhile the plausible mechanisms of apoptotic effects were characterized by

DAPI, annexin-V FITC and gene involved including Caspase9, Caspase3, Bax and Bacl2. The overall results manifested that the A2058 cell proliferation has stopped along with increasing the concentration of alcoholic extract of *Ficus carica*. DAPI staining and annexin assay revealed that increased percentage of apoptotic cells in treated cells and alcoholic extract leads to breakdowns in Chromatin The results of real-time PCR revealed elevated gene expression of Caspase9, Caspase3, Bax, and decreased Bcl2 treated with the extract. Our findings showed that *Ficus carica* leaf alcoholic extract causes A2058 cancer cells to undergo apoptosis.

Keywords: *skin cancer, Ficus carica, apoptosis, A2058 cell line, Melanoma.*

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Introduction

There are over 100 types of cancer, and its causes range from infections to genetic factors, making it one of the most concerning diseases that affect people. Cancer conjures up terrifying visions of suffering, deformity, and unavoidable death (Baumann, et al., 2018). Cancer can be characterized as the absence of

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regular growth control. When normal stability of the organization of tissues and organs are disturbed, a variety of diseases will arise (Howell, et al., 2017). Most people are discovered when the illness has progressed. lowering cancer's incidence and death rate and Improving cancer patients' quality of life is one of the objectives of the National Cancer Control Program (White, et al., 2017).

There is increasing evidence to indicate The prevention of cell death may result in cancer. These are obtained mainly through natural evidences, like the translocations found in leukemias and lymphomas (Liu, et al., 2017). For a few genes involved in cell death, transgenic and knockout mice have demonstrated that cancer can result from a failure of cell death. Although a number of research have been conducted to ascertain the expression levels of genes for cell death inhibitors in different forms of cancer, these investigations only offer circumstantial data (Wang, et al., 2017). Taking this into consideration, it would be useful instead to design synthetic compounds or to discover newer natural compounds that can induce apoptosis in cancer selectively allows that to differentiate between the normal and cancer cells (Mansoori, et al., 2019).

Since herbal medicines are generally thought to have fewer negative effects when applied to humans, there has been a resurgence of interest in using them to treat a variety of illnesses in recent years (Huang, et al., 2017). The potential to prevent cancer in herbal plants have been suggested as one of the mechanism for the observed beneficial impacts (Yu, et al., 2018).

Numerous studies stated that consumption of medicinal plant and herbal supplements, considerably reduces the oxidative stress activity and elevates the antioxidant and anticancer potential (Ekeleme-Egedigwe, et al., 2019; Kumar, et al., 2020; Vallejo, Salazar, & Grijalva, 2017; Forni, et al., 2019). Research laboratory experiments have shown that the biological properties of herbal medicine due to the capability of natural bioactive compounds including phenolic, flavonoids (Imran, et al., 2019). Several researches specify that natural constitutes of herbal medicine prevents development of cancer *via* numerous probable mechanism, reducing the inflammatory activity and attenuating the oxidative stress condition (Venkateswara Rao, et al., 2017). Medicinal plants as the agents that fight cancer are studied for their apoptogenic activities and most investigations screened potential anti-cancers using established methods and further understanding the underlying mechanisms attributed to apoptosis (Tungmunnithum, et al., 2018). In this study, the promising properties of *Ficus carica* leaf extract as an alternative treatment for skin cancer were studied. Furthermore, the potential mechanisms underlying Skin cancer and the cytotoxic effects of *Ficus carica* leaf extract (A2058 cell line) were examined in more detail using flow cytometry analysis and gene expression.

Materials and Method

Chemical

In the present research the skin cancer (A2058 cell line) were acquired from the Iran Cell Bank at the Pasteur Institute. Medium (RPMI) from Roswell Park Memorial Institute, Trypsin/EDTA (1X), Penicillin-Streptomycin-Glutamine (PEN-STREP, 100X) and Trypan Blue solutions were purchased from Bio idea, Iran. FBS was purchased from GIBCO, USA. PBS tablets, MTT powder and DAPI were procured from Sigma, USA. Annexin V-FITC Apoptosis Staining / Detection Kit (ab14085) was obtained from ABCAM. The cDNA Synthesis Kit and RNA Extraction Kit and SYBR Green came from Pars Tous, Iran.

Extraction Procedure

In this purpose, 3 gr of dried powder of *Ficus carica* was mixed with 30 ml of methanol and placed in the dark for 72 hours at ambient temperature. Three times a day throughout this time, the mixture was thoroughly mixed. Following a 72-hour period, Whatman filter paper was used to filter the mixture, and

the solvent was then vacuum-evaporated. Then, the extract was placed in the oven at temperatures between 40 and 50 °C for 24-48 hours (Hatware, et al., 2018).

Anticancer Properties

The anticancer potential of extracts were assessed by using MTT assay. In short, A2058 cells were cultivated in RPMI medium and kept in a humidified 5% CO2 incubator at 37°C. 100 µL of cells (5 × 103 /100 µL) were grown in a 96-well cell culture plate (Greiner Bio-One, Germany) and incubated for 24 hours at 37°C with 5% CO2. Flasks containing cells were observed daily to investigate cell growth and density, as well as the possible occurrence of bacterial and fungal infections. To prepare the main stoke for the treatment, 0.001 g of the extract was weighed and dissolved in 50 µl dimethyl sulfoxide. Then, it was brought to a volume of 1 ml with the culture medium. Following the substitution of RPMI medium with RPMI containing varying extract quantities, cells were incubated at 37°C in a humidified 5% CO2 incubator. Finally, the viability of the cells was determined using the MTT assay. This analysis was performed in triplicate (Sajjadi, et al., 2019).

DAPI Protocol for Fluorescence Imaging

DAPI is a fluorescent dye that strongly binds to the rich areas of adenine and thymine bases in DNA. First, coverslips (25 mm, sterile) were fitted in a 6-well plate, then SKOV-3 cells (5×10^4 cells) were gently added onto the coverslip. In each well, the culture media was added, which included the control and treatment groups. After 24 hours, the supernatants were removed and methanol (1 mL) was applied to fix the cultured cells. Then, the cells were washed 3 times in PBS. After that, enough DAPI stain solution was added to cover the cells. The dish was positioned in an incubator and shielded from the illumination. At last, the cells were washed 2 times in PBS and the cell morphology was assessed by a fluorescent microscope (Wu, et al., 2019).

Annexin V-FITC Apoptosis Staining

ABCAM detection kit was used to investigate the apoptosis of the A2058 cells in the control and treatment groups. After 24 hours of the cell culture, the cells were treated with IC50 concentrations. After another 24 hours, the cells were separated and centrifuged at 2000 RPM and ambient temperature for 5 min. After removing the supernatant, each well received 500 µl of Annexin V binding buffer 1X. Then, FITC Annexin V (5 µl) and 5 µl of propidium iodide were added to the wells. Finally, the treated groups were incubated for five minutes in the dark and analyzed by flow cytometry (Sheeba, Ali, & Anuradha, 2020).

Apoptosis-Related Genes Analysis

Due to examine the gene profiling involve in apoptosis the RNA extracted from the A2058 cells. Phosphate-buffered saline (PBS; 0.1 M, pH 7.2) was used to rinse and scrape the cultivated cells. After being scraped, the cells were collected after centrifuging for ten minutes. at 3000g. After removing the supernatant, RNA was extracted from the cells. Following the kit's instructions, the RNA extraction was carried out (RNA extraction kit; Cinna Gene, Iran). The reverse transcript polymerase chain reaction (PCR) technique was accustomed to instantly transform the total RNA into complementary DNA (cDNA) using the cDNA Synthesis K. Changes in gene expression in the control and treatment groups of the A2058 cells were analyses by Bio-Rad CFX96 RT-PCRs and Bio-Rad CFX manager software. The primer sequences that were used in Real-time PCR listed in Table 1.

Table 1. Primer Characteristics Used for the Gene Expression Study

Gene	Sens	Antisense
GAPDH	TGACTTCAACAGCGACACC	TTGCTGTAGCCAAATTCGTT
Caspase 3	AGACAGACAGTGGTGTTGATG	GTTCATCCAGTCGCTTTGTGC
Caspase 9	CCAGAGATTCGCAAACCAGAG	CAATGTGAACTTCTGCCGTGA
P53	TTGCCGTCCCAAGCAATGGA	TCTGGGAAGGGACAGAAGATG

Results and Discussion

Anticancer Activity Analysis

The outcomes of the anticancer properties of *Ficus carica* on skin cancer (A2058 cell line) are presented in Figure 1. The obtained results indicated that the *Ficus carica* the extract significantly ($P < 0.05$) and dose-dependently reduced the growth of cells. The IC_{50} values of extract was found at 550 µg/ml. This finding is an agreement with study of *Ficus carica* extract that inhibited the proliferation of promyelocytic leukemia cells (HL-60) (Zubair, et al., 2016). Another investigation conducted by Zhang et al indicated that *Ficus carica* leaf extract had strong anticancer potential against the MDA-MB 231 cell line and enhance the upregulated of Bax and P53 genes in breast cancer (Zhang, et al., 2018).

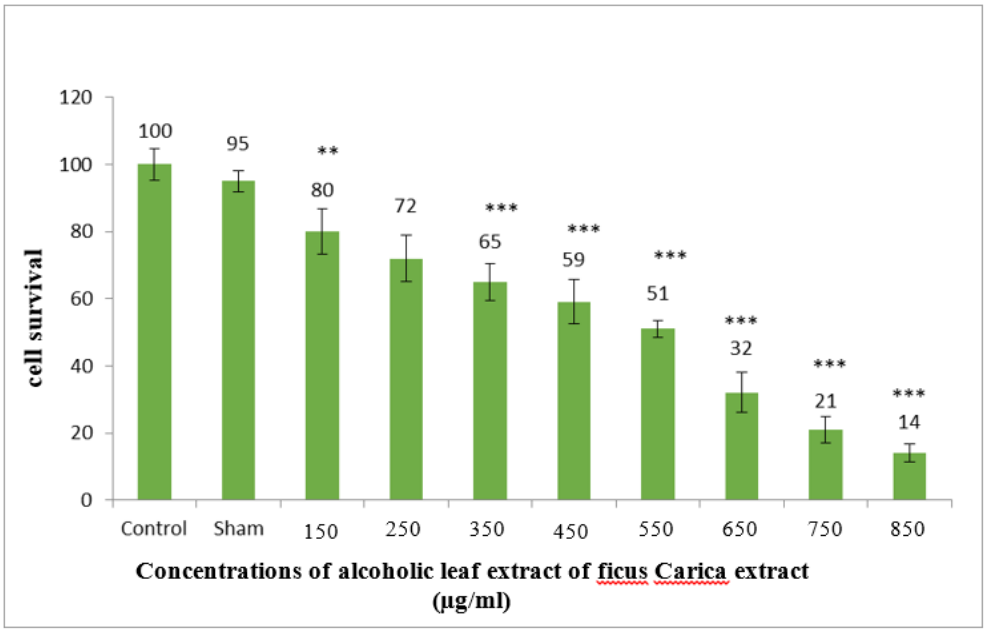

Figure 1. Cell Survival Results of Leaf Extract of *Ficus carica* Extract

Apoptosis assessment by DAPI staining

DAPI labelling was used to assess the induction of apoptosis and track the morphological alterations of the cell nucleus following treatment. When compared to round, less strongly labelled reference cells, Figure 2 clearly shows the prominent feature of apoptosis in all treatment groups. In the meantime, the results showed that the control group's healthy cells' nuclei were uniform and integrated, while the cells treated with the leaf extract indicate apoptosis and showed DNA fragments (see Figure 2).

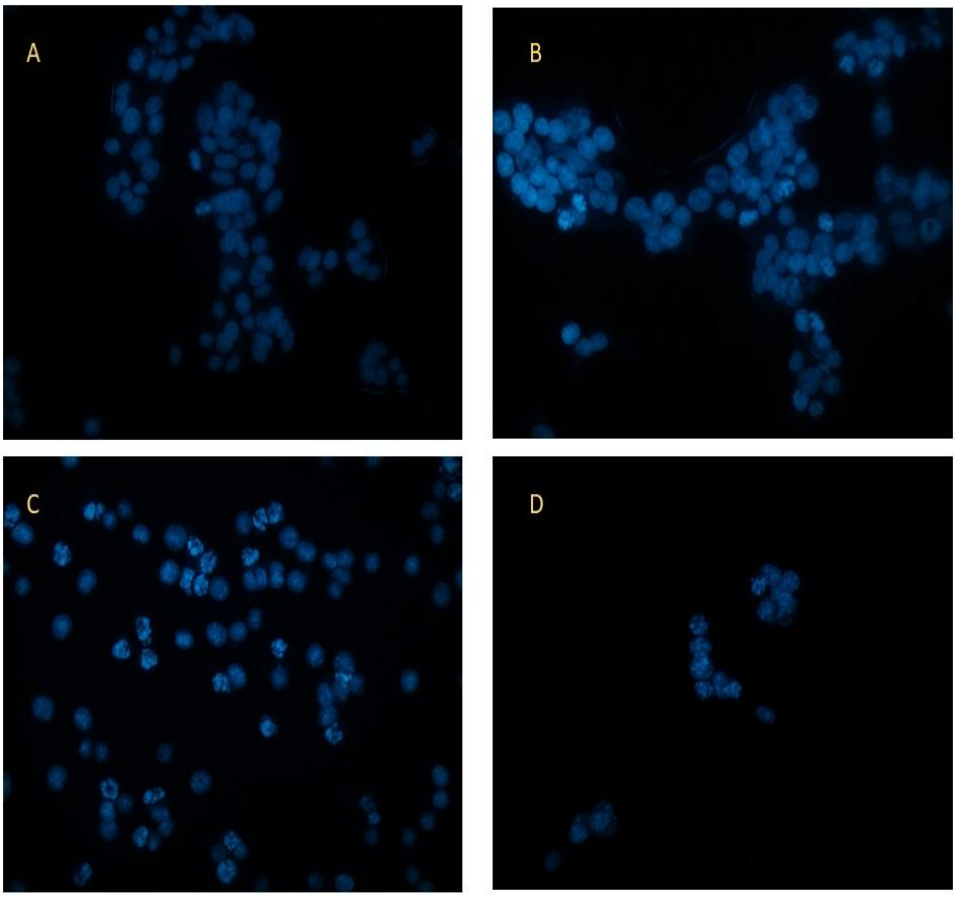

Figure 2. DAPI Staining of (a) Control, (b) the Leaf Extract with Respective Concentration of 250 µg/ml (b), 550 µg/ml (c) and 850 µg/ml. (d)

Annexin V-FITC Apoptosis Analysis

A flow cytometric study verified the DAPI staining's observation of apoptotic cell death. In this regards, the A2058 cell were treated with three different concentrations of *Ficus carica* (250, 550 and 850 µg/ml) and results display that 93% of the cells were survive in control group meanwhile, the cells were treated with 250 and 550 µg/ml were survived 89.4% and 65% respectively that indicate the low apoptosis had been happen. The apoptotic cell was significantly increased to 43.7% at concentration of 850 µg/ml of plant extract compare with control group: The outcomes of the cytotoxicity test and DAPI staining were in agreement with these findings. Similar research on the effects of black turtle bean extract on breast cancer revealed that after 50 µg/ml for 48 hours, the percentage of apoptotic cells was 15.97 and 60.7% for MCF-7 and MDA-MB231 cells; after 100 µg/ml for 48 hours, the percentage of apoptotic cells was 94.47 and 70.34%, respectively, indicating that apoptosis was the primary mechanism of cell death (Kumar, et al., 2017). Many studies have confirmed the importance of using molecular techniques to detect various pathogens, including parasitic ones (Oleiwi, Alrikaby, & Soud, 2022).

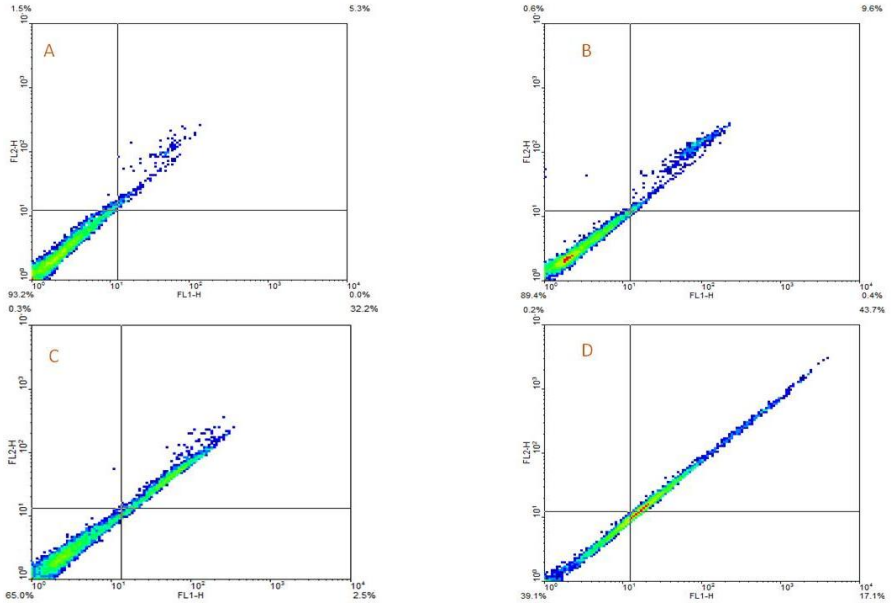

Figure 3. Flow Cytometry Analysis of (a) Control, (b) the Leaf Extract with Respective Concentration of 250 µg/ml (b), 550 µg/ml (c) and 850 µg/ml (d)

Gene Expression Analysis

The expression of genes linked to apoptosis, such as Bax, Bcl2, and caspase 3and Caspase 9 in skin cancer A2058 cell line were evaluated with Real-Time PCR (Figure 4). The overall results exhibited that the expression of Bax, Capsase 3, Caspase 9 genes upregulated and the Bccl2 genes downregulated after treatment with different concentration of leaf extracts. These results illustrated that the different concentration of *Ficus carica* extract could alter the bax/bcl2 ratio and activated the caspase-3 and 9 pathway-mediated apoptosis.

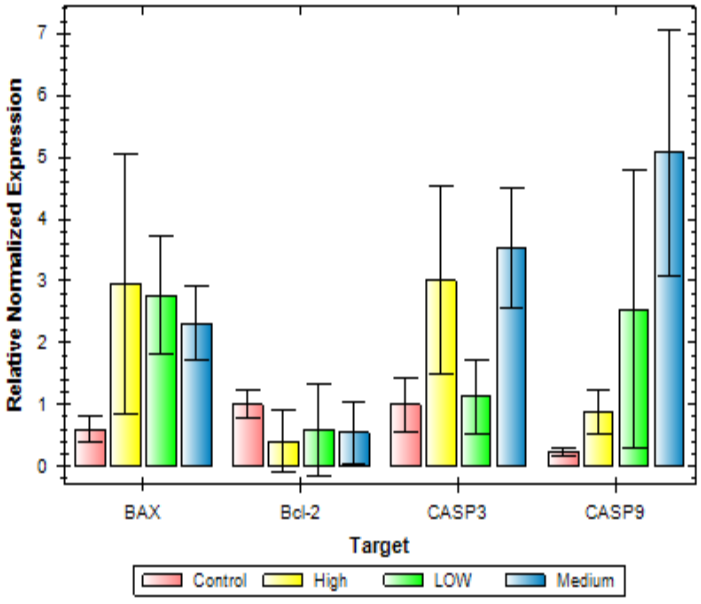

Figure 4. Real-Time PCR of the Bax, Bcl2, Caspase 3 and and Caspase 9 (Red: control, Yellow: 850 µg/ml of *Ficus carica* Extract, Green: 250 µg/ml of *Ficus carica* Extract, Purple: 550 µg/ml of *Ficus carica* Extract)

Conclusion

Our results demonstrated that the *Ficus carica* extract has substantial anticancer properties and suppressed growth. Furthermore, the apoptotic cell death was validated by the flow cytometry and microscopic analysis. Through the genes involved, *Ficus carica* extract at varying concentrations could suppress proliferation mediated by apoptosis, according to gene expression profiling. According to the study's findings, *Ficus carica* extract may be a viable substitute anticancer medication for the treatment of skin cancer.

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