EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 70, 3295:3303, OCTOBER, 2024
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY
Submit Date : 05-07-2024 • Accept Date : 29-08-2024 • Available online: 01-10-2024 • DOI : 10.21608/edj.2024.301911.3103

ANTICANCER EFFECT OF ARTICHOKE AND FENNEL SEEDS EXTRACTS ON ORAL SQUAMOUS CELL CARCINOMA CELL LINE: IN VITRO STUDY

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ABSTRACT

Objectives: The purpose of this study was to evaluate the cytotoxic and apoptotic effect of artichoke and fennel extracts as a natural agent against OSCC cell line (OECM-1).

Methods: Two hundred gm of Artichoke leaves were soaked in ethanol solution 50% for 48 h at 4 °C to obtain ethanolic extract. One hundred fifty gm of Fennel seeds were soaked in 1.5 L in ethanol solution 75% for 48 h at 4 °C. Calculation of IC_{50} for each group was determined based on the concentration-response curves of analyzed cellular metabolic activity. Then the exact concentration of IC50 was used. Assessment of the apoptosis in cancer cells was done by staining the cell lysate using the ***Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit*** for flowcytometry, ***Catalog. V13241, Invitrogen, ThermoFisher, UK***.

Results: The study revealed a cytotoxic effect of Artichoke at IC50 concentration (0.425±0.032µg/mL) and (0.769±0.041µg/mL) for Fennel seeds, and a high significant difference between the IC_{50} of the two extracts ($t=11.52$, $p<0.0003$). Meanwhile, a highly significant difference was detected between the % of apoptotic cells after treatment with Artichoke and Fennel seeds compared to untreated cells ($p<0.0001$), no significant difference was detected between the % of apoptotic cells in OSCC treated with Artichoke when compared to cells treated with Fennel seeds.

Conclusion: Both artichoke and fennel seeds induced apoptosis and had cytotoxic impacts on the cancer cells. They could become a promising field for further research in cancer therapy.

KEYWORDS: Artichoke, Fennel seeds, OSCC, Cytotoxicity, Apoptosis

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INTRODUCTION

Oral cancer is considered a major health problem that affects any age. There is low public awareness, and many patients present with advanced stage, resulting in high rate of mortality. About 90% of oral malignancies are squamous cell carcinomas, and two-thirds of instances occur in underdeveloped nations [(1)].

Primary therapeutic modalities for treatment of oral squamous cell carcinoma (OSCC) are surgical intervention, radiotherapy and chemotherapy. Chemotherapeutic agents play a critical role in handling the treatment of malignant tumors yet they have cytotoxic effect on the adjacent normal cells thus, several researches were studying the effect of an alternative naturally occurring drug with minimal side effects and maximal efficacy in cancer therapy [(2)] .

Nowadays the use of traditional medicine is widely used, different types of plants contain large sources of natural antioxidants that might help for the development of new drugs [(3)]. Also, Chemo preventive agents is a promising method that relies on natural products that provide protection against reactions caused by oxidation and provide vital preventative measures such as induction of apoptosis, epigenetic processes modification and suppression of cell proliferation [(4)].

Several epidemiological studies done in recent years have revealed that fruit and vegetable-rich diets induce apoptosis and have anticarcinogenic properties. Apoptosis is the main type of programmed cell death that occurs in all cells to maintain homeostasis and cellular integrity. Cancer treatment methods target this mechanism by promoting apoptosis in cells for preventing cancer [(5,6)].

Artichoke is a very popular plant commonly farmed in the Mediterranean area for its nutritional and therapeutic properties [(7)]. Artichoke-derived compounds are widely known for their antioxidant, anticancer and antifungal activities, the extracts from the plant's leaves and edible section are thought to have anticancer potential against several forms of cancer due to the presence of some components including flavonoids, polyphenols, inulin and fatty acids which were found to be cytotoxic against several cancer cells such as breast, colon, skin, hepatocellular carcinoma and leukemia[(8)].

Hassabou and Farag assessed the anticancer effect of artichoke extract on OSCC, their study revealed that artichoke extract caused reduction in cell viability and initiation of apoptosis [(9)]. Simsek and Uysal showed that Artichoke extracts inhibited the proliferation and also induced apoptosis of a human colorectal cancer cell line [(10)]. Also, Villarini. M et al., conducted their study on colon cancer cell line by using artichoke extract and revealed its effect on cancer cells by induction of apoptosis [(11)].

Fennel seed (Foeniculum vulgare) is a tiny greenish-brown seed from the Umbelliferon family. It grows throughout the Mediterranean and Western Asia. It is a popular spice in Egyptian cuisine and traditional medicine due to its estrogenic, diuretic, and antioxidant properties that makes studies very essential to detect its anticarcinogenic potential. It possesses a promising source of natural antioxidants such as vitamins C and E, oleoresins and phenolic compounds. Also, other components that show biological activities such as antidiabetic, anti-inflammatory and hepatoprotective effects[(12)]. Consequently, Fennel extracts were chosen in our study to determine its influence on OSCC.

Kaveh.R et al. revealed the antioxidant and anticancer effect of the fennel extract against lung cancer cells by increasing the cell cytotoxicity [(13)]. Also Zaahkouk.S et al showed the anticancer effect of fennel seeds extract by inducing DNA damage on breast, colon and liver cancer cells [(14)].

The present study was done to evaluate the cytotoxic and apoptotic effect of artichoke and fennel extracts as a natural agent against OSCC cell line.

MATERIALS AND METHODS

1-Preparation of natural extract

Two hundred gm of artichoke leaves were dried in oven, cut into pieces then soaked in 2 L of ethanol solution 50% for 48 h at 4 °C then the mix was filtered two times by using filtering paper to obtain ethanolic extract [9]. The ethanolic extract was collected and evaporated to dryness under vacuum at 40 C and stored at 4°C until when needed. One hundred fifty gm of dried fennel seeds were washed two times with distilled water and then soaked in 1.5 L in ethanol solution 75% for 48 h at 4 °C then the mixture was filtered twice using filtering paper. The ethanolic extract was collected and evaporated to dryness under vacuum at 40 C [15,23].

2- Preparation of stock solution of Natural extracts

A readymade solution of 100μg/mL was prepared by reconstitution of the 0.1gm in the appropriated volume of 1 mL of dimethyl sulfoxide (DMSO), then sonication is done for 5 seconds and aliquoted and kept at -20°C until needed. In each experiment, the final concentrations of the test compound by diluting the stock with the medium. The carrier solvent (0.1% DMSO) was added to the control cells.

3. Detection of the half-maximal cytotoxic effect (IC_{50}) of Artichoke and Fennel seeds on OSCC cells

Before the experiment, The OSCC cell line (OECM-1) were planted into a 96-well culture plate. 8×10^3 cells per well in 200 μL of *Dulbecco's Modified Eagle Medium* (DMEM), supplemented with 1% of *penicillin G sodium* (10.000 UI), 10% *Fetal Bovine serum* (*FBS),* and *streptomycin* (10 mg) and *amphotericin B* (25 µg) (***Gibco, Thermosientific, Germany***). To attach cells, incubation of culture plates was done at 37 degrees Celsius in a 5% CO2 atmosphere for 24 hours.

On the following day, serial concentrations of each natural extract "0.01, 0.1, 1.0, 10, 100μg/mL[14], were prepared for cells treatment. The concentrations were selected to cover a wide range of concentrations for the used compounds. Also, for the control cells the carrier solvent (0.1% DMSO) was used. A positive control was used known as Cisplatin with a concentration of 10.0 μmol/mL. Cells were preserved at 37°C in an atmosphere of 5% CO2 for 48 hours [16].

The final step of incubation, cytotoxic assay of the cells was done using the ***Vybrant® MTT Cell Proliferation Assay Kit***, cat no: M6494 (***Thermo Fisher, Germany***). A 100μL of media was discarded and switched by new media. 20 μL of *4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (MTT) solution (1mg/mL) (***Invitrogen, ThermoScientific, Germany)*** was included to each separated well. Incubation of the plates was done at 37°C and 5% CO2 for four hours. Finally, the MTT solution was discarded, and 100 µL of sodium dodecyl sulfate with hydrochloric acid *(SDS-HCL)* was inserted into the wells. Cell viability was determined by measuring the optical density at 570 nm on a spectrophotometer (***ELx 800; Bio-Tek Instruments Inc., Winooski, VT, USA***).[17].

After conducting the cell proliferation assay, determination of the % of cell viability was done, representing the cytotoxic effect of serial doses of extracts at each test. The relationship between the log dose of (inhibitor) and the normalized response was done by XY curve. Linear regression analysis was used to determine the best-fit point.

The IC50 was estimated with the GraphPad Prism 9 software, version 9.1.0(221). Calculation of IC_{50} for each group was determined based on the concentration-response curves of analyzed cellular metabolic activity, which were standardized to untreated OSCC cells. The IC50 calculated was 0.428μg/mL for the Artichoke and 0.768 μg/mL for the Fennel seeds.

4. Assessment of Apoptosis in OSCC cells after treatment with IC_{50} of Artichoke or Fennel seeds for 48 hours.

Prior to the experiment's day, OSCC cells were cultured as mentioned before. On the next day, the

culture media was discarded and replaced by new media, followed by treatment with the previously calculated IC50 " 0.428μg/mL for the Artichoke and 0.768 μg/mL for the Fennel seeds.

Cells treated with PBS, present the negative control group (NC). After treatment, incubation of the cancer cells was done for 48 hours at 37 °C in an atmosphere of 5% CO2.

Collection of cells was done using 0.25% Trypsin EDTA, then twice washing with PBS was done and the cell pellet was suspended in 1 mL of DMEM media after adjusting the count to 1x10^6 cells/mL.

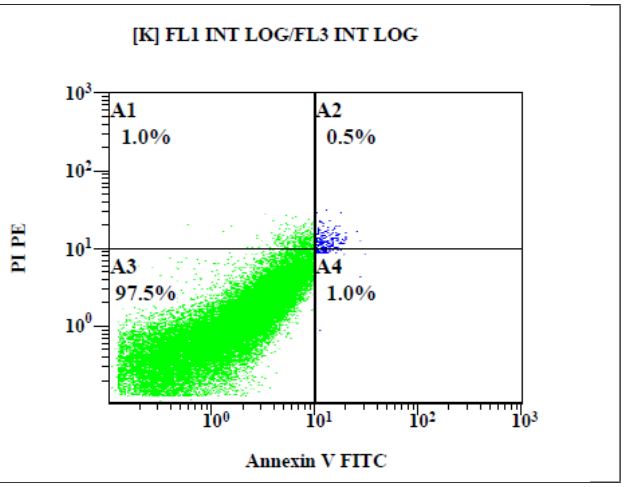
To assess the apoptosis in cancer cells, the cell lysate was stained using the ***Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit*** for flowcytometry, ***Catalog. V13241, Invitrogen, ThermoFisher, UK***. Centrifugation at 400xg for 5 minutes was used twice for washing the cells pellet. The supernatant undergoes aspiration and elimination. 10^6 of cells were resuspended in 1X annexin-binding buffer "adding 0.1 mL of 5X annexin binding buffer to 4 mL deionized water, and the cells incubated at room temperature for 5 minutes. A 5 µL of Alexa Fluor 488 annexin V and 1 µL 100 µg/mL propidium iodide (PI) working solution was added to each 100 µL of cell suspension. The stained cells were incubated at room temperature for 15 minutes.

Addition of 400 µL 1X annexin-binding buffer was done after the incubation period and mixed slowly then the samples were kept on ice. Immediately, analyzation of the stained cells was done by flowcytometry and the fluorescence emission at 575 nm and 530 nm was measured by using 488 nm excitation.

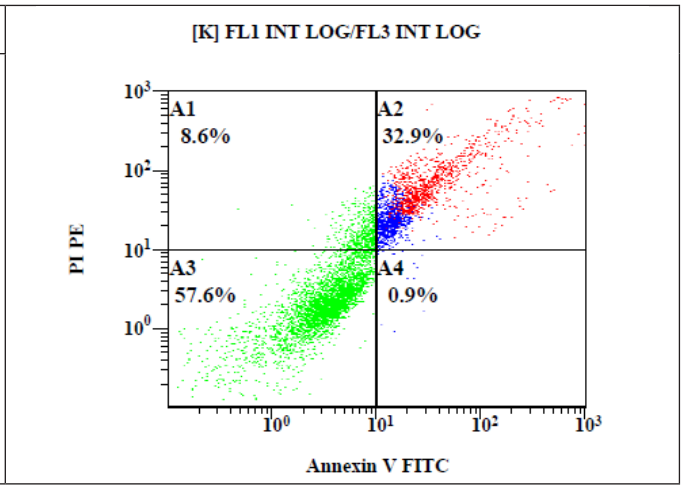
The ***Beckman Coulter Navios EX software***: ***SM: BE14548 software version: Navios EX. (Beckman Coulter)*** was used to analyze flow cytometry data. Upon analysis, the cells separated according to their labeling into three categories: (1) living cells (Annexin V^-/PI^-) cells, (2) early apoptotic cells (Annexin V^+, PI^-) cells, (3) late apoptotic cells (Annexin V^-, PI^+), and (4) necrotic (dead) (Annexin V^+/PI^+) cells. The monoclonal antibodies determine the externalization of phosphatidylserine in apoptotic cells using recombinant annexin V which is conjugated to green, fluorescent FITC dye and dead cells using propidium iodide (PI). Necrotic cells showed red fluorescence when stained with Propidium iodide stains, dead cells showed green and red fluorescence, apoptotic cells showed green fluorescence and live cells showed little or no fluorescence after treatment with both probes. The Flow cytometry histograms are presented in **Table 1.**

TABLE (1) Distribution of cancer cells (OSCC) according to their staining with Annexin/PI: necrosis (n) (%)

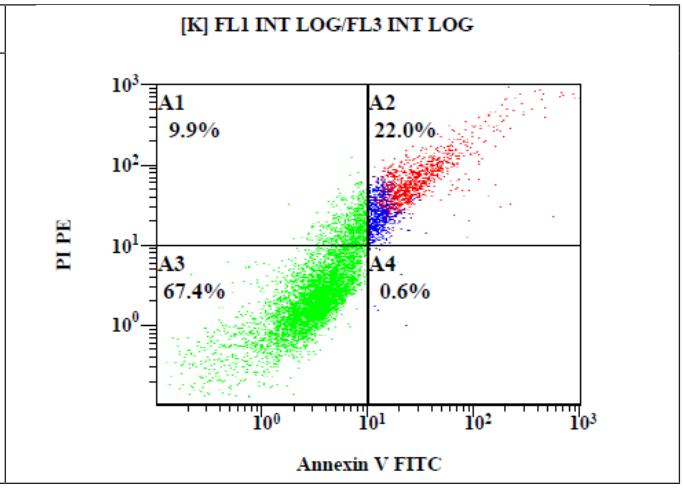
Untreated OSCC cells	
Alive cells (%)	97.5%
Early Apoptotic cells (%)	1.0%
Late apoptotic cells (%)	1.0%
Necrotic cells (%)	0.5%



OSCC cells treated with IC_{50} of Artichoke	
Alive cells (%)	57.6%
Early Apoptotic cells (%)	0.9%
Late apoptotic cells (%)	8.9%
Necrotic cells (%)	32.9%



OSCC cells treated with IC_{50} of Fennel seeds	
Alive cells (%)	67.4%
Early Apoptotic cells (%)	0.6%
Late apoptotic cells (%)	9.9%
Necrotic cells (%)	22.0%



Apoptotic cells: late apoptotic and Necrotic cells: strongly expressed Propidium iodide (red), highly express Annexin V (green), living cells are negative for both Annexin V and PI.

Statistical Methods & analysis for data:

The comparative analysis between the alive, apoptotic and necrotic cells was evaluated using the ANOVA test followed by A post-hoc test. The post-hoc test was employed to investigate variations across various groups. Moreover, independent t-test was employed to assess differences between IC50 of the two extracts. P-value: significance level, $p>0.05$: non-significant level, $p<0.01$: high significant, $p<0.05$: statistically significant.

RESULTS

1- Comparative analysis between the determined IC_{50} of Artichoke and Fennel seeds on OSCC cells

After treatment of cells with serial concentrations of each extract for 48 hours, the obtained results revealed a high cytotoxic effect of Artichoke at a lower concentration of (0.425±0.032µg/mL), compared to 0.769±0.041µg/mL for Fennel seeds, and a high significant difference between the IC_{50} of the two extracts ($t=11.52$, $p<0.0003$) (**Figure 1**).

2- Comparison between the % of OSCC living, apoptotic and necrotic cells after treatment with IC_{50} of Artichoke or Fennel seeds for 48 hours

The obtained results revealed a lower percentage of living OSCC cells associated with treatment with

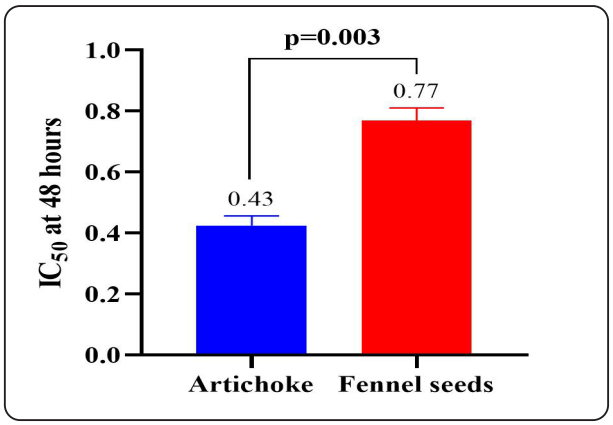


Fig. (1) Bar chart illustrating a significant difference (p=0.003) between the IC50 of Artichoke and Fennel seeds on OSCC after serial concentrations treatment of each extract for 48 hours.

of Artichoke, followed by cells treated by Fennel seeds for 48 hours, compared to untreated cells (p<0.001). The percentage of living and apoptotic cells was determined by Annexin V/PI staining by Flow cytometry. These percentages reflected the alive, apoptotic, and necrotic cells in cells treated with IC50 of the compound.

Meanwhile, a highly significant difference was detected between the % of apoptotic cells after treatment with Artichoke or Fennel seeds compared to untreated cells (p<0.0001), no significant difference was detected between the % of apoptotic cells in OSCC treated with Artichoke when compared to cells treated with Fennel seeds (p>0.05). **Table 2**.

Furthermore, a higher percentage of necrotic cells were associated with the treatment of Artichoke, followed by cells treated with Fennel seeds, compared to untreated OSCC (p<0.001). In addition, a highly significant difference was detected between the two treated groups regarding the % of living or necrotic cells. **Table 2**, **Figure 2**.

TABLE (2) Comparative analysis between the percentage of living, apoptotic, and necrotic cells in different studied groups.

Group	OSCC	OSCC treated with 0.428µg/mL of Artichoke	OSCC treated with 0.768 µg/mL of Fennel seeds	Statistics (ANOVA)
Alive	97.6±0.557	60.3±4.27*	66.6±0.917*	F: 186.7, p<0.0001
Apoptotic "early & late"	2.10±0.361	9.80±0.65*	10.7±0.265*	F: 319.2, p<0.0001
Necrotic	0.49±0.07	32.7±0.45*	23.6±1.6*/#	F: 901, p<0.0001

****: statistical significance compared to untreated OSCC (p<0.01) #: statistical significance compared to OSCC treated with 0.428µg/mL of Artichoke for 48 hours (P<0.01).***

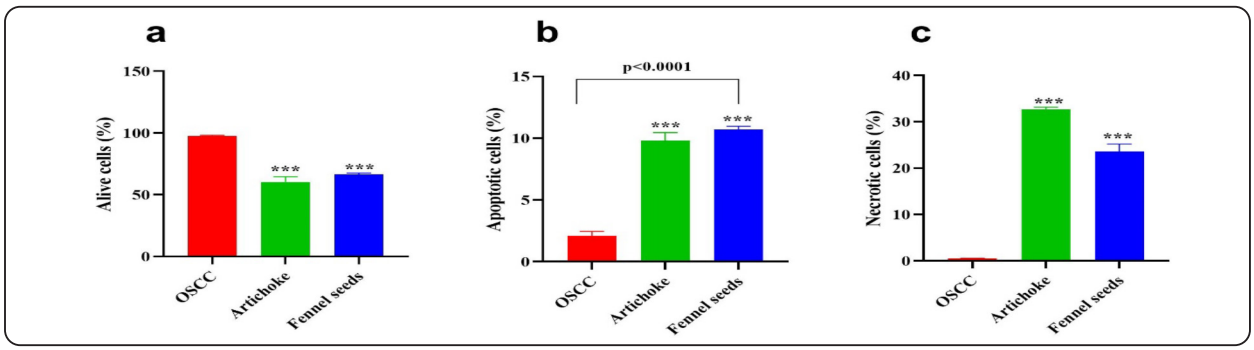


Fig. (2) Boxplot graph illustrating the significant difference for the living (a), apoptotic (b), and necrotic cells (c) between the OSCC treated either with Artichoke or Fennel seeds, in comparison to untreated cells tested. Data are showed as mean ± SD, *: shows a significant difference (p<0.01) in comparison to the negative control group, #: indicates a significant difference (p<0.01) compared to the cells treated with Artichoke for 48 hours.

DISCUSSION

The mainstays of cancer treatment include surgery, radiotherapy, and chemotherapy; however, a combination of these modalities yields superior results. Unfortunately, these therapies have harmful side effects such as vomiting, hair and weight loss, hormonal disorder, immunodeficiency nausea and fatigue[18].

Plant-derived products have emerged as a novel therapy option for cancer due to their high efficacy, accessibility, and almost complete lack of side effects when compared to synthetic drugs [19].

Currently, functional foods like grains, spices and herbs have acquired interest as anticancer agents due to their significant amounts of bioactive compounds that can contribute to inhibition of the multi-step processes of carcinogenesis [20]. Thus, in this study we examined the effect of artichoke and fennel seeds on OSCC cell lines (OECM-1).

Our results revealed a high cytotoxic effect of Artichoke on OSCC cell line. Meanwhile, a high significant difference was detected between the percent of apoptotic cells after treatment with Artichoke compared to untreated cells.

These results were in agreement with a study done on OSCC cell line showed a decrease in cell viability and initiation of apoptosis and was explained that the cell cycle was arrested at G2/ M phase. Additionally the defects in the G2/M arrest checkpoint permit the damaged cell to enter mitosis and undergo apoptosis, this improves the increase of the cytotoxic effects of the extract [9]. Also, Vígh S et al. who reported in their study that artichoke extract comprehends some active substances as flavonoids and polyphenols that serve as an excellent anticancer agents against breast cancer by inducing apoptosis on the cancer cells [15].

Our results were also consistent with the study of Pulito.C et al. who proved that artichoke extract induced apoptosis against mesothelioma cell line by activating the cleavage of caspase-7 and caspase-3 and poly-ADP ribose protein. Furthermore, they stated that artichoke were found to be rich in chlorogenic acid, cynaropicrin and caffeoyl quinic acids all of which have potent anticancer activity [21].

Similarly, Villarini.M et al conducted their study on colon cancer by using artichoke extract and showed that artichoke rich in chlorogenic and caffeoylquinic acids that were able to induce mitochondrial activation dependent pathway of apoptosis [11].

Likewise, it has been shown by Noriega Rodríguez.D et al, that phenolic antioxidants components mainly mono- and dicaffeoylquinic acids of artichoke increased cell apoptosis on colon and breast cancer cell line [22]. Regarding fennel seeds, our study showed that the cytotoxic effect of fennel seeds on OSCC cell line was lower than artichoke extract, but it also induced apoptosis in cancer cells compared to untreated cells.

These findings were also concordant with Kaveh R et al. 2023 who revealed that Fennel seed extract had a cytotoxic effect against lung cancer cells [13]. Moreover, Zaahkouk.S et al, Showed the anticancer effect of fennel seeds extract by inducing DNA damage on breast, colon and liver cancer cells and inducing expression of p53 in cancer cell lines treated by IC50 of fennel seeds extract more than untreated cancer cell lines [14].

Comparable results were also reported by Ke.W et al et al, who conducted their study using extract of fennel seeds extract on hepatocellular cancer cells and showed that it decreased cell viability, stimulated apoptosis, and effectively prevented cell migration in hepatocellular cancer cells via inducing activation of caspase-3 and apoptosis [23]. A study done on five different lymphoblastic cell line showed that Fennel seeds contain small amounts of polyacetylene which caused cytotoxicity [24].

In addition to Ghasemian. A et al, in their study on breast cancer cell line they reported that

fennel seeds had a cytotoxic effect and induced apoptosis [25]. Another study done on breast and liver cancer cells showed that Fennel seeds extract had an effective role in anticancer effectiveness by increasing the antioxidant protection mechanism, lipid peroxidation modulation and inhibitory influence on free radicals [26].

Sokkar et.al showed in their study that artichoke induce apoptotic pathways via the up and downregulation of the pro-apoptotic Bax gene and the anti-apoptotic gene Bcl2, respectively. DNA fragmentation results involved in their study show a 5-folds increase in Bax expression, while apigenin showed an elevation of 2.9-folds only. On the other hand, a decrease in Bcl2 expression level was observed revealing that the extracts induce programmed cell death by removing the anti-apoptotic Bcl2 barrier. Artichoke extracts were also found to upregulate the cyclin-dependant kinase inhibitor as well as p21[19].

CONCLUSION

Artichoke and fennel seeds could become a good anti-carcinogenic agent. Through the triggering of apoptosis, they both have a cytotoxic and inhibiting effect on the proliferation of cancer cells. They may possibly become applicable for the supplementations of certain antitumor therapies. Further studies are required to reveal the exact mechanism of their cytotoxicity and apoptosis. Furthermore, the effect of combination of the two extracts determines whether they have synergistic effect.

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