

INVESTIGATION OF THE APOPTOTIC EFFECT OF CARDAMOM OIL ON LARYNGEAL CANCER CELL LINE (A NON-RANDOMIZED IN VITRO STUDY)

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ABSTRACT

Aim: To evaluate the apoptotic effect of Cardamom oil on laryngeal cancer cells in comparison to Doxorubicin which could give insight for a new approach of therapy for Head and Neck cancer.

Methods: A subcultured Hep-2 cell line was placed into five study groups: a control group, a group exposed to Cardamom oil for 24 hours, another group subjected to Cardamom oil for 48 hours and the other two groups treated with DOX for the same time intervals. The percentage of cell viability was measured using MTT assay. Apoptosis analysis was assessed using Flow Cytometry represented by dot plots. Apoptosis indicator BAX was also measured by means of ELISA assay for each group. Furthermore, cells were morphologically examined using the inverted phase microscope.

Results: Cardamom oil reduced the percentage of viable cells with increasing dose. A gradual increase in the percentage of dead cells in the Cardamom oil and DOX treated groups was revealed with increasing time of treatment from 24 hours to 48 hours. Levels of BAX were elevated in all treated groups of Cardamom oil and DOX with the highest level observed in DOX (48 hours) group and the lowest levels recorded in the control group. Moreover, cultured cells under the inverted phase microscope showed signs of apoptosis which increased as duration of treatment increased.

Conclusions: Cardamom oil has a time-dependent apoptotic effect on Head and Neck Squamous Cell Carcinoma, and exerts its action through enhancement of BAX which is a crucial molecule in the apoptotic process.

KEYWORDS: Apoptosis, Cardamom, Bax, Laryngeal, carcinoma

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INTRODUCTION

Cancer is a major health concern and is considered the second leading cause of death worldwide. Surgical resection, radiation, and chemotherapy are the standard of care for the treatment of cancer, nonetheless, emergence of resistance remains a challenge⁽¹⁾. Most of the diagnosed head and neck malignancies, known as head and neck squamous cell carcinoma (HNSCC), originate from the mucosal epithelium of the oral cavity, pharynx, and larynx⁽²⁾. This aggressive tumor has a fatal prognosis despite the use of numerous treatment protocols⁽³⁾. One of the most prevalent HNSCC is laryngeal squamous cell carcinoma (LSCC) with an estimated 184,615 newly diagnosed cases and mortalities of 99,840 patients in 2020. Usually, stage I tumors have the most favorable prognosis, unlike advanced stage LSCC which has a very low survival rate⁽⁴⁾. The five-year rate of survival has decreased from 66% to 63% over the past 40 years. In the ongoing fight against head and neck malignancies, greater emphasis is now being placed on developing novel therapeutic strategies⁽⁵⁾.

Fortunately, a number of epidemiological studies have demonstrated a link between a diet high in vegetables, fruits, herbs, and spices and a lower incidence of most malignancies, making these foods effective chemopreventive agents⁽⁶⁾. Cardamom (*Elettaria Cardamomum Maton*), a dietary phytoproduct, is known as queen of spices because of its very pleasant aroma⁽⁷⁾. It is an old herb, indigenous to India, Pakistan, Burma and Bangladesh⁽⁸⁾. Cardamom essential oil is the concentrated extract of Cardamom spice and it is believed that active ingredients found in Cardamom's essential oils have many medical properties including lowering blood pressure, improving liver health, fighting bacterial infections as well as sedative activities^(9, 6). Moreover, it exerts chemopreventive, anti-oxidant and anti-inflammatory potential against cancer⁽¹⁰⁾.

Recent advances in cancer research are focused on the development of new agents that target the execution of apoptosis⁽¹¹⁾ since the induction of apoptosis in cancer cells is considered the primary objective of cancer therapy⁽¹²⁾. Pre-clinical and clinical trials have investigated the therapeutic effect of many potential agents, regarding apoptotic efficiency^(13,14).

Thus, it is beneficial to examine the apoptotic activity of Cardamom oil and compare it to the apoptotic activity of a well-known broad spectrum antitumor agent, Doxorubicin (DOX) on laryngeal cancer cells using state of the art techniques as Microculture Tetrazolium (MTT) assay, Enzyme-linked Immunosorbent Assay (ELISA), Flow cytometry and H&E staining.

MATERIAL

Laryngeal squamous cell carcinoma cell line Hep-2 was obtained from Nawah Scientific Research Center, Cairo, Egypt and stored in liquid nitrogen container at (-196°C).

Whole green Cardamom pods were purchased from the Ministry of Agriculture, Cairo, Egypt. Extraction of Cardamom oil was done at Nawah Scientific Research Center, Cairo, Egypt.

Doxorubicin (DOX) was purchased from Sigma Aldrich (Munich, Germany).

DRG® Human BCL2 Associated X (BAX) ELISA (EIA-4487) (96 wells) was purchased from DRG International, Inc. (Springfield, USA).

The following reagents were also included:

- BAX Microtiter Plate, One Plate of 96 Wells, A plate using break-apart strips coated with a mouse monoclonal antibody specific to BAX.
- BAX Antibody, 11 mL, a yellow solution of biotinylated monoclonal antibody to BAX.

METHODS

Study Design

Laryngeal cancer cell line was placed into five study groups: a control group, a group exposed to Cardamom oil for 24 hours, another group subjected to Cardamom oil for 48 hours and the other two groups treated with DOX for the same time intervals. The percentage of viable cells was measured by MTT assay and IC₅₀ was detected for both treatments. Apoptosis analysis was assessed for each group using Flow Cytometry represented by dot plots. Apoptosis indicator BAX was also measured by means of ELISA for each group. Furthermore, cultured cells were morphologically examined using the inverted phase microscope to detect signs of apoptosis in each group.

1-Extraction of Cardamom oil

Extraction was carried out using 200gm of whole green Cardamom pods. These pods were then transferred into a round bottom flask and around 700ml of distilled water was added. Heat energy was provided to the round bottom flask via heating mantle. Initially round bottom flask was heated to 60°C and then the temperature was gradually increased from 60°C to 80°C, 80°C to 90°C and finally to 100°C. The extraction was carried out till no more drops of oil were coming out of the condenser. ⁽¹⁵⁾

2-Measurement of Cell Viability by MTT Assay ⁽¹⁶⁾

1. Before the MTT assay, one ml of cells (50,000 to 100,000 cells/ml) were plated into each well of 96-well culture plates for 24 hr. Then, the cells were incubated for 24 hr in CO₂ incubator.
2. Experimental media were removed, and cells were washed in PBS, after treatment of cells with antibiotic and antifungal for 24-72 hr.
3. Cardamom extract and DOX were separately added for cell line with serial dilutions

(1000µg/ml, 100µg/ml, 10µg/ml, 1µg/ml, 0.1µg/ml and 0.01µg/ml). ⁽¹⁷⁾

4. Cells were continuously examined under the inverted phase microscope, and then incubated with medium containing 0.5 mg/ml MTT in CO₂ incubator at 37°C for 4 hr.
5. The medium was aspirated, and the formazan product was solubilized with DMSO.
6. Absorbance at 570 nm was measured for each well using a microplate reader (FLUOstar Omega).

The results were interpreted and the half maximal inhibitory concentration (IC₅₀) was estimated as follows:

The mean optical density (OD) of each column of the 96-well plates was calculated by dividing the sum of OD of the column wells by the number of wells.

The mean OD of each column with specific concentration treatment was divided by the mean of the control untreated cells to get the percent of viability and cytotoxicity of each concentration treatment.

A graph was plotted between the log concentration on the X axis vs % viability on the Y axis.

A curve was plotted between them for each study group as shown in **Fig. (1)**.

3-Analysis of apoptosis by Flow Cytometry

This assay was used to count the number of cells that have undergone apoptosis. Apoptosis was detected by initially staining the cells with Annexin V and PI solution followed by Flow Cytometry analysis. It is based on the principle that normal cells are hydrophobic in nature as they express phosphatidyl serine (PS) in the inner membrane (side facing the cytoplasm) and when the cells undergo apoptosis, the inner membrane flips to become the outer membrane, thus exposing phosphatidyl serine.

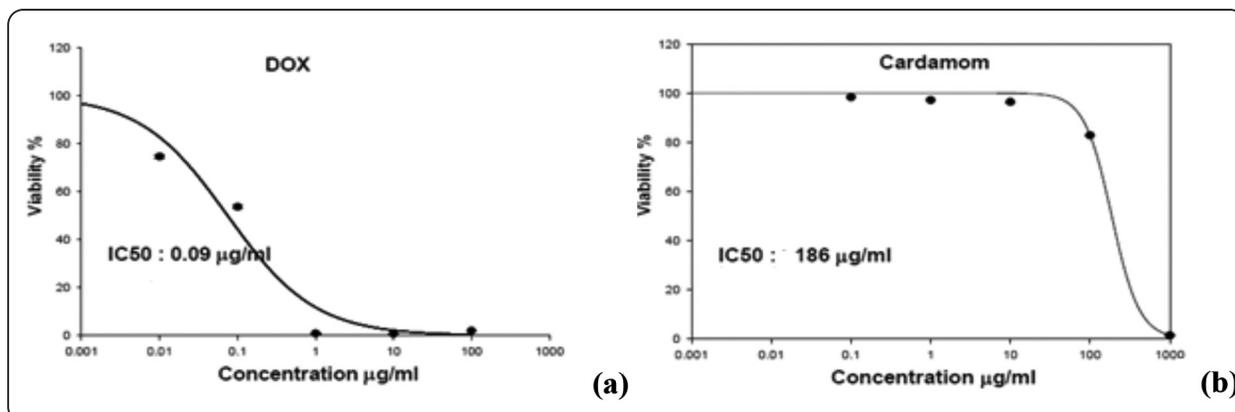


Fig. (1) Two graphs showing curves between the log concentration of (a) DOX and (b) Cardamom oil versus the viability % of cancer cells.

The exposed PS is detected by Annexin V, thereby marking apoptotic cells. On the other hand, PI stains necrotic cells as it has the capability to intercalate into the leaky DNA, this helps to differentiate between apoptotic and necrotic cells.⁽¹⁸⁾

Cardamom oil and DOX were added to flasks with IC50 concentration and were incubated for time periods of 24 hours and 48 hours.

Analysis was performed by BD FACS Caliber (Bioscience, flow cytometer, United States) at a rate of 100-200 cells/sec, with data displayed as dot plots.

4- BAX Activity Assay using ELISA:

ELISA is a test that uses antibodies and color change to identify certain antigen. A specific antibody was applied to the sample so it could bind to the antigen; this antibody was linked to an enzyme. In the final step, a substance containing the enzyme's substrate was added to obtain a reaction which produced a detectable color change in the substrate.⁽¹⁹⁾

5- Hematoxylin and Eosin Staining (H&E):

Cells were stained by H&E and they were examined using the inverted phase microscope to detect signs of apoptosis.

Statistical Analysis:

Values were presented as mean and standard deviation (SD) values. Data were explored for normality using Kolmogorov-Smirnov test of normality. The results of Kolmogorov-Smirnov test indicated that data were normally distributed (parametric data), therefore independent t test was used for 2 groups comparison. One way analysis of variance (ANOVA) and Tukey's post hoc tests were used for comparison of more than 2 groups. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with SPSS 18.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) for Windows.

RESULTS

Cell Viability and Cytotoxicity Determination:

The cell viability percent showed a gradual decrease with increasing doses of the proposed treatments (Cardamom oil and Doxorubicin (DOX)). Values were calculated after 72 hours and the IC50 value which is the half maximal inhibitory concentration needed to inhibit a biological process by half and it was determined as the following: DOX: **0.09 µg/ml** and Cardamom: **186 µg/ml**. These 2 doses were the doses used in the next assays at two periods 24 hours and 48 hours.

Apoptosis analysis using Flow Cytometry

Dot plot representation of flow cytometric analysis showed that the highest percentage of viable cells was detected in the control group (98.14 % viable cells) followed by Cardamom oil (24 hours) group (89.97% viable cells) then Cardamom oil (48 hours) group (86.33%) then DOX (24 hours) group (85.27%) and the least percentage of viable cells was found in DOX (48 hours) group (83.46%)

(Fig. 2). An increase in percentage of cells in early apoptotic phase (located in the second quadrant) and late apoptotic phase (located in the third quadrant) was observed in Cardamom oil and DOX groups when treatment duration lengthens compared to the control group. On the other hand, necrotic cells (located in the fourth quadrant) were also detected with the highest percentage in Cardamom oil and DOX groups at time interval of 48 hours (Fig. 2).

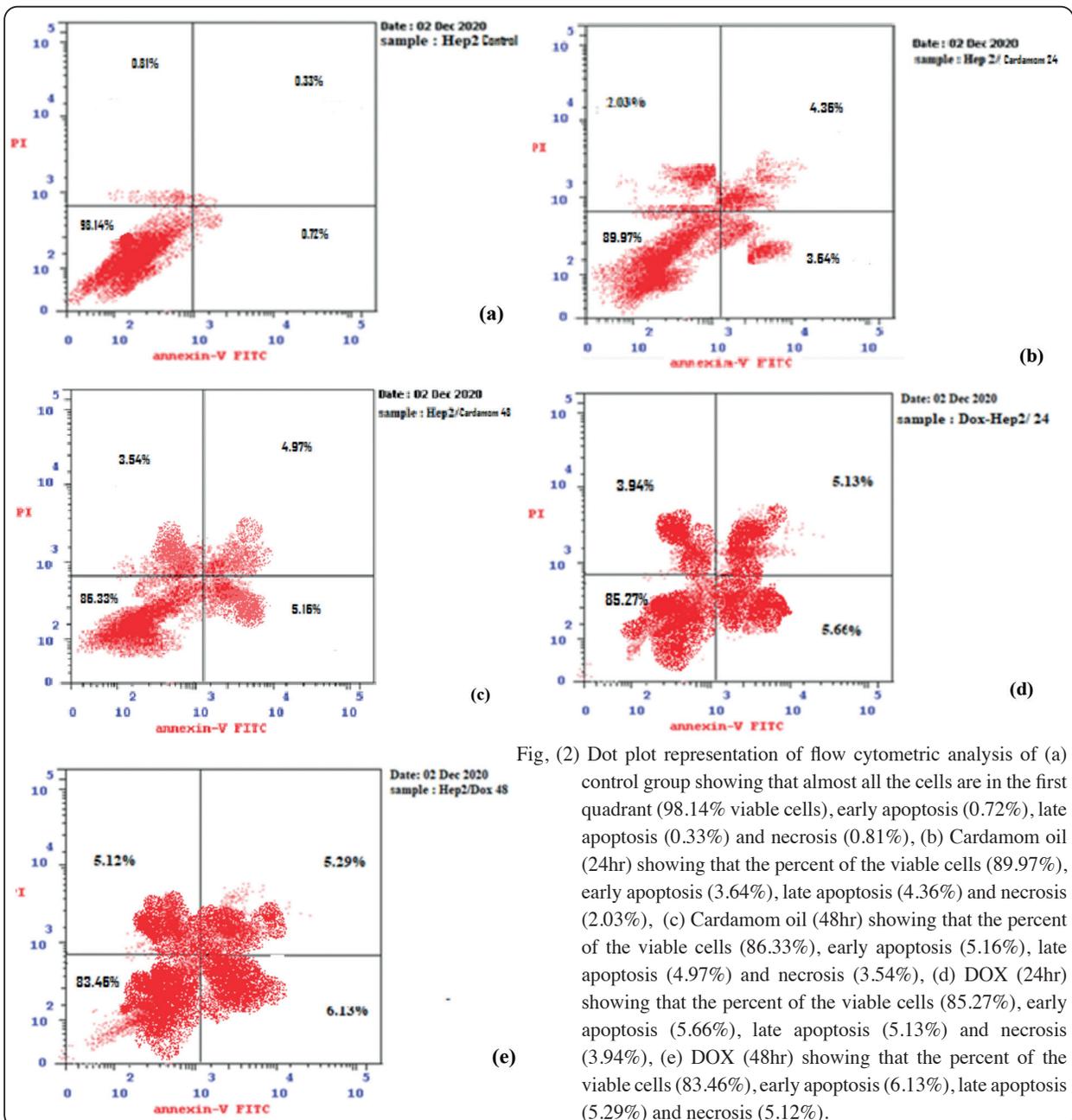


Fig. (2) Dot plot representation of flow cytometric analysis of (a) control group showing that almost all the cells are in the first quadrant (98.14% viable cells), early apoptosis (0.72%), late apoptosis (0.33%) and necrosis (0.81%), (b) Cardamom oil (24hr) showing that the percent of the viable cells (89.97%), early apoptosis (3.64%), late apoptosis (4.36%) and necrosis (2.03%), (c) Cardamom oil (48hr) showing that the percent of the viable cells (86.33%), early apoptosis (5.16%), late apoptosis (4.97%) and necrosis (3.54%), (d) DOX (24hr) showing that the percent of the viable cells (85.27%), early apoptosis (5.66%), late apoptosis (5.13%) and necrosis (3.94%), (e) DOX (48hr) showing that the percent of the viable cells (83.46%), early apoptosis (6.13%), late apoptosis (5.29%) and necrosis (5.12%).

Statistical Analysis:**Regarding total dead cells%, early apoptosis%, late apoptosis%, and necrosis%:**

The highest mean value was obtained in DOX (48 hours) group, followed by DOX (24 hours), then Cardamom oil (48 hours) group, then Cardamom oil (24 hours), with the lowest percentage shown in the control group. ANOVA test revealed that the difference between groups was statistically significant ($P=0.00$) (Table (1), Fig. (3)).

BCL2 Associated X (BAX) Gene Expression by Enzyme-linked Immunosorbent Assay (ELISA):

BAX expression level increased with increasing time of exposure of cells to Cardamom oil from

24 hours to 48 hours. The same increase in BAX expression was shown in cells subjected to DOX however the increase was higher in case of DOX treatment. The least expression level was seen in the control group (Table (2), Fig. (4)).

Statistical Analysis:

The highest mean value was recorded in DOX (48 hours) group, followed by DOX (24 hours) group, then Cardamom oil (48 hours) group, then Cardamom oil (24 hours) group, with the least value recorded in control group.

ANOVA test revealed that the difference between groups was statistically significant ($P=0.00$).

TABLE (1) Descriptive statistics and comparison of Total Dead Cells analysis in different groups (ANOVA test)

Groups	Total Dead Cells	Early apoptosis	Late apoptosis	Necrosis
Cardamom extract/24hr	10.03±0.9 ^b	3.64±0.7 ^b	4.36±0.7 ^a	2.03±0.3 ^c
Cardamom extract/48hr	13.67±1.1 ^{ab}	5.16±1.3 ^a	4.97±0.6 ^a	3.54±0.4 ^{bc}
DOX/24hr	14.73±1.27 ^{ab}	5.66±1.2 ^a	5.13±0.6 ^a	3.94±0.4 ^b
DOX/48hr	16.54±1.52 ^a	6.13±1.2 ^a	5.29±0.4 ^a	5.12±1.1 ^a
Control Hep-2	1.86±0.2 ^c	0.72±0.1 ^c	0.33±0.02 ^b	0.81±0.03 ^d
P value	0.000*	0.000*	0.000*	0.000*

Significance level $P \leq 0.05$, *significant. Difference between values of the same column (values of the 5 groups) is significant. Tukey's post hoc test: Different superscript letters in the same column are significant.

TABLE (2) Descriptive statistics and comparison of BAX gene in different groups (ANOVA test)

Groups	pg/mL	Fold change
Cardamom extract/24hr	218.7d±13.2	4.6d±0.1
Cardamom extract/48hr	275.8c±10.4	5.8b±0.2
DOX/24hr	298.65b±12.9	6.3a,b±0.3
DOX/48hr	325.24a±15.5	6.86a±0.4
Control Hep-2	47.43e±2.9	1±0
P value	0.00*	0.00*

Significance level $P \leq 0.05$, *significant. Difference between values of the same column is significant. Tukey's post hoc test: Different superscript letters in the same column are significant.

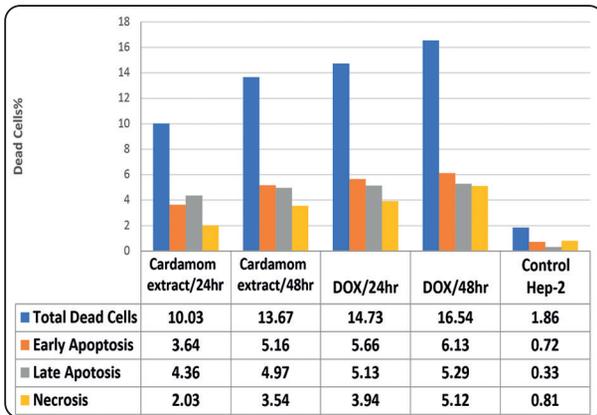


Fig. (3) Bar chart illustrating Total Dead Cells % among different groups

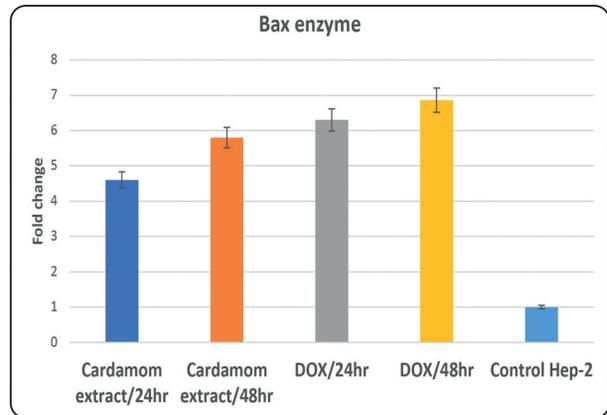


Fig. (4) Bar chart demonstrating mean BAX gene fold change among group

Morphological assessment

Cells were regularly checked under inverted phase microscope to ensure viability. Cultured cells of the Hep-2 cell line showed signs of cytotoxicity with marked decrease in number of viable cells in treated groups from 24hr to 48hr. (Figs. (5-9)).

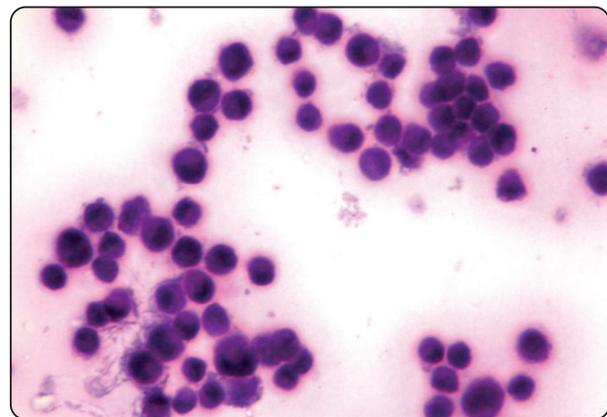


Fig. (5) A photomicrograph of Hep-2 cell line control group showing regular cells with hyperchromatic nuclei and nuclear pleomorphism (H & E, Original magnification 100X, Oil).

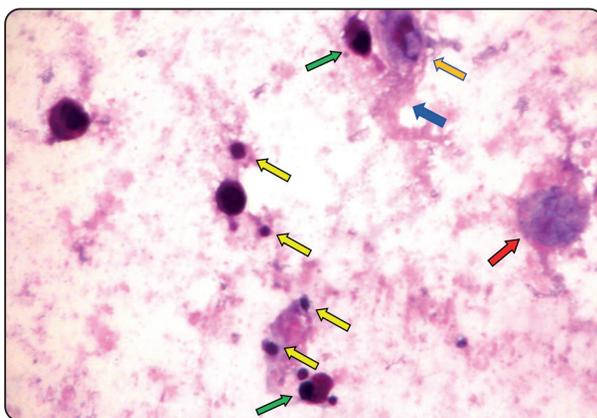


Fig. (6) A photomicrograph of Hep-2 cell line treated with Cardamom oil after 24hr showing start of cell shrinkage (Green arrows), Horseshoe nuclei in early apoptosis (Orange arrow), apoptotic bodies (Yellow arrows), necrotic cells and ruptured cell membrane (Red arrow) and cell debris (Blue arrow) (H & E, Original magnification 100X, Oil).

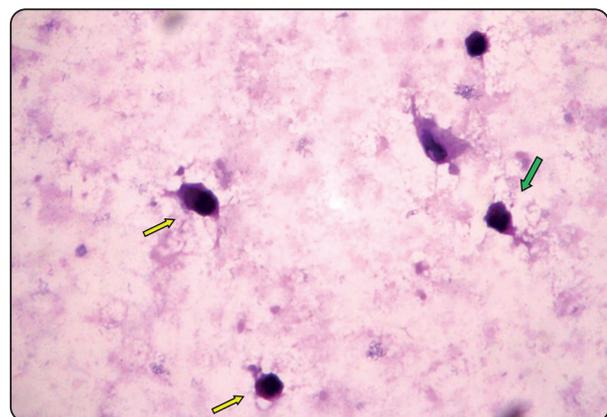


Fig. (7) A photomicrograph of Hep-2 cell line treated with Cardamom oil after 48hr showing shrunken apoptotic cells (Yellow arrows) with irregular cell membranes (Green arrows) (H & E, Original magnification 100X, Oil).

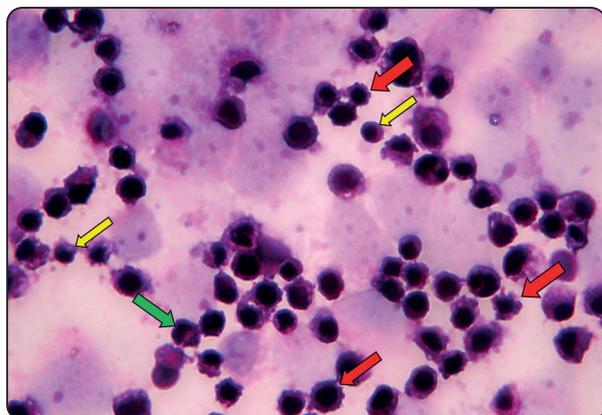


Fig. (8) A photomicrograph of Hep-2 cell line treated with DOX after 24hr showing shrunken apoptotic cells (Yellow arrows) with irregular cell membranes (Red arrows) and peripheral condensation of chromatin (Green arrow) (H and E, Original magnification 100X, Oil).

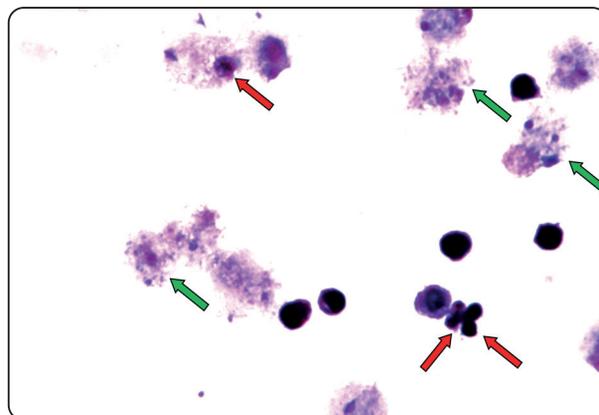


Fig. (9) A photomicrograph of Hep-2 cell line treated with DOX after 48hr showing shrunken apoptotic cells with shrunken nuclei (Red arrows) and apoptotic bodies (Green arrows) (H and E, Original magnification 100X, Oil).

DISCUSSION

Head and neck squamous cell carcinoma (HNSCC) is considered a major public health concern, thus, an urgent need for novel treatment approaches is required in order to achieve organ preservation, higher survival rates and more efficient therapies⁽²⁰⁾. Advances in biotechnology, drug development, and molecular approaches in the present century were expected to provide improved outcomes for patients with HNSCC. However, despite these advances, outcomes are still mostly unchanged for the past few decades and treatment-associated hazards are still ongoing⁽²⁾. Accordingly, HNSCC was the lesion of choice in this study.

Fortunately, some spices show an anticancer potential by inducing apoptosis and inhibiting proliferation, invasion and metastasis. By using spices in combination with chemotherapy to achieve similar cancer control, the doses of the medications were reduced, which lessened their negative effects on healthy cells. Thus, combined treatment is a potential therapeutic approach for cancer and more natural components should be studied, and their detailed mode of action should be clearly investigated⁽⁷⁾. Studies have shown the anticancer and antioxidant effect of Cardamom in

many types of cancer such as colon⁽²¹⁾, breast⁽¹⁰⁾, and skin cancer⁽⁶⁾. In this study, the apoptotic effect of Cardamom oil on laryngeal cancer cells was investigated in comparison to a well-known chemotherapeutic drug, Doxorubicin (DOX) to be able to judge the apoptotic effect of Cardamom oil.

The findings of our study showed a notable increase in the percentage of total dead cells in Cardamom oil groups when compared to the control group and when treatment duration lengthens. These results are in line with **Kong et al (2019)**⁽²²⁾ who stated that cardamom enhances apoptosis in breast cancer. Also, **Badroon et al (2020)**⁽²³⁾ reported that cardamom increased the percentage of total dead cells in hepatocellular carcinoma in a time dependent manner. The increase in the percentage of total dead cells in Cardamom oil groups was slightly lower than DOX groups, this is consistent with **Freitas et al (2020)**⁽²⁴⁾ who also used DOX as a positive control against Squamous cell carcinoma (SCC) and found that DOX exerts apoptosis of a large number of cells of SCC.

In the current study, the level of BCL2 Associated X (BAX) was significantly increased in Cardamom oil cells when compared to the control group and when treatment duration lengthens from 24 hours

to 48 hours which demonstrates an active apoptotic process. In the same context, **Lu et al (2018)**⁽²¹⁾ stated that cardamom significantly inhibits chemotherapy-resistant colon cancer cell growth, induces apoptosis and promotes the activation of BAX expression. They explained the mechanism through suppression of NF- κ B protein expression. Similarly, Cardamom ingestion in DMBA-treated mice blocked NF- κ B activation and reduced cyclooxygenase-2 expression which reduced both the size and the number of skin papillomas generated on the skin due to the DMBA treatment. It was hence suggested that cardamom has a potential to act as a chemopreventive agent to prevent papillomagenesis on the skin.⁽⁶⁾ Also, **Jou et al (2015)**⁽²⁵⁾ reported that Cardamom exerts potent anti-proliferative and apoptosis inducing activities on oral squamous cell carcinoma (OSCC) through the involvement of the mitochondrial apoptotic pathway and decreasing the mitochondrial membrane potential. Our finding augments the theory that activation of BAX, which triggers apoptosis, is how cardamom oil exercised its anti-cancer effect. Levels of BAX was higher in DOX treated cells than in Cardamom oil treated cells with the highest increase in DOX group at 48 hours, this is in accordance with **Sharifi et al (2015)**⁽²⁶⁾ who stated that DOX markedly increases the levels of BAX against breast cancer in time dependent manner. This result lends credence to the idea that cardamom's anti-cancer effect was brought about via activating BAX, a crucial molecule in apoptosis regulation.

CONCLUSION

In conclusion, our study revealed that Cardamom oil has a potential apoptotic effect on HNSCC in a time-dependent manner. These promising findings could pave the way for future research into using complex therapy with Cardamom oil and chemotherapeutic drugs on oral squamous cell carcinoma (OSCC). We recommend additional animal trials as well as clinical trials to confirm the anti-cancer effect of Cardamom oil and its possible use for OSCC.

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