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ASSESSMENT OF ANTICANCER EFFECT OF CARDAMOM ON ORAL SQUAMOUS CELL CARCINOMA CELL LINE (A NON-RANDOMIZED IN VITRO STUDY)

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ABSTRACT

Aim: To investigate the anticancer effect of Cardamom extract on Hep-2 cell line which may aid in development of a novel treatment modality to Head and Neck Squamous Cell Carcinoma.

Methodology: Hep-2 cell line was divided into five groups: one control group, two groups treated with Cardamom extract, and another two treated with Doxorubicin, each of the two treatments was applied for 24 and 48 hours. Then, cellular viability was measured using microculture tetrazolium assay, cell cycle analysis was done using Flow Cytometry and eventually, apoptotic activity was evaluated using Real time polymerase chain reaction to measure the fold change for caspase-3 enzyme.

Results: Cardamom extract succeeded to decrease the percentage of viable and proliferating cells with increasing dose. On the other hand, it increased the percentage of apoptotic cells and levels of caspase-3.

Conclusions: Cardamom extract has a potential cytotoxic effect on Head and Neck Squamous Cell Carcinoma cell line in a dose and time dependent manner, and exerts this action through induction of apoptosis, and its action is comparable to Doxorubicin action.

INTRODUCTION

Head and Neck Squamous Cell Carcinoma (HNSCC) can arise from subsites within the oral cavity, oropharynx, hypopharynx, larynx, and nasopharynx [(1)]. Almost 650,000 new cases of head and neck cancer and 300,000 deaths occur world-wide each year[(2)]. Oral cancer is a common cancer worldwide, and 90% of oral cancers are squamous cell carcinoma (SCC).

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The main therapeutic choices of oral squamous cell carcinoma (OSCC) are surgery, radiotherapy, and/or chemotherapy. Even with the numerous basic clinical research and reports that have emerged, the overall 5-year survival rate for patients with OSCC is still poor [3]. Fortunately, numerous epidemiological studies have shown that a diet rich in vegetables, fruits, herbs and spices is associated with a reduced risk of most cancers, and thus serve as potent chemopreventive agents [4].

Cardamom has different therapeutic effects including regulating blood pressure, diuretic, and sedative activities. Moreover, it demonstrates chemopreventive, anti-oxidative, anti-proliferative, and apoptotic activities against non-melanoma skin, breast and colon cancers [5,6,7]. One of the most powerful chemotherapeutic agents is Doxorubicin (DOX) as it is a broad spectrum antitumor agent. DOX is used in the treatment of solid tumors in adult and pediatric patients. Many studies have shown its anticancer effect against HNSCC and OSCC [8,9,10]. Thus, It seems interesting to study the possible anticancer effect of Cardamom Extract and compare it to the anticancer effect of DOX on SCC using state of the art techniques as Microculture Tetrazolium (MTT) essay, Flow Cytometry and Real Time Polymerase Chain Reaction (RT-PCR)

MATERIAL

Cell line of squamous cell carcinoma Hep-2 was purchased from Nawah Scientific Research Center, Mokattam, Cairo, Egypt. Also, Extraction of Cardamom oil was done at Nawah Scientific Research Center (Mokattam, Cairo, Egypt). Doxorubicin (DOX) was purchased from Sigma Aldrich (Munich, Germany).

METHODS

Hep-2 cell line was divided into five groups: one control group, two groups treated with Cardamom extract, and another two treated with Doxorubicin, each of the two treatments was applied for 24hr and 48hr. Then, cellular viability was measured using microculture tetrazolium assay, cell cycle analysis was done using Flow Cytometry and eventually, apoptotic activity was evaluated using Real time polymerase chain reaction to measure the fold change for caspase-3 enzyme.

RESULTS

1- Cell viability

The cell viability percent showed a gradual decrease with increasing doses of the proposed treatments (Cardamom extract and DOX).

2- Cell cycle analysis

(a) In G0-G1 phase, a gradual decrease in the amount of cells entering the cell cycle was observed among the groups with increasing time of treatment. Regarding the **S phase**, the Cardamom extract treated groups at 24hr and 48hr periods showed a gradual decrease in the amount of cells from one group to the other with increasing time. Similarly, this was observed when comparing the DOX treated groups. Unlike the **G0-G1** and **S phases**, a gradual increase in the amount of cells in **G2-M phase** with increasing time was detected in Cardamom extract and DOX treated groups at different periods of time (24hr and 48hr).

Statistical analysis

Statistical analysis revealed high statistical significant difference among the Cardamom extract and DOX treated groups at 24hr and 48hr periods in G0-G1, S phase and G2-M phase **(figure 1).**

(b)Apoptotic phase

A gradual increase in the amount of apoptotic cells in the Cardamom extract and DOX treated groups was revealed among the two periods of time.

Statistical analysis

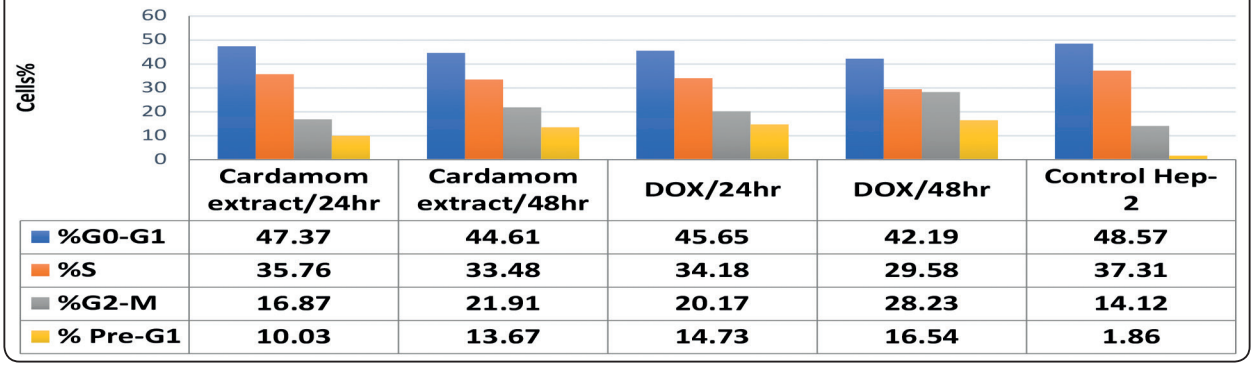
The gradual increase in the amount of apoptotic cells showed a high statistical significant difference among the Cardamom Extract and DOX groups.

(c) Analysis of DNA histogram and Dot Plots:

The percentage of apoptotic cells showed an increase with Cardamom extract and DOX treatment to Hep-2 cell line. The highest values were observed in Cardamom extract and DOX at 48hr period 13.67% and 16.54% respectively. However, they were almost undetectable in the control group. Apoptotic cells were almost equally distributed between early and late apoptotic phases in all the treated groups **(figure 2).**

3- Caspase-3 Enzyme Expression by Real Time Polymerase Chain Reaction (RT-PCR):

Levels of caspase-3 were elevated in all treated study groups of Cardamom extract and DOX with increasing time of treatment. The highest concentration was observed in DOX (48hr) group.



	Cardamom extract/24hr	Cardamom extract/48hr	DOX/24hr	DOX/48hr	Control Hep-2
%G0-G1	47.37	44.61	45.65	42.19	48.57
%S	35.76	33.48	34.18	29.58	37.31
%G2-M	16.87	21.91	20.17	28.23	14.12
% Pre-G1	10.03	13.67	14.73	16.54	1.86

Fig. (1): Bar chart illustrating Hep-2 cells % in different groups

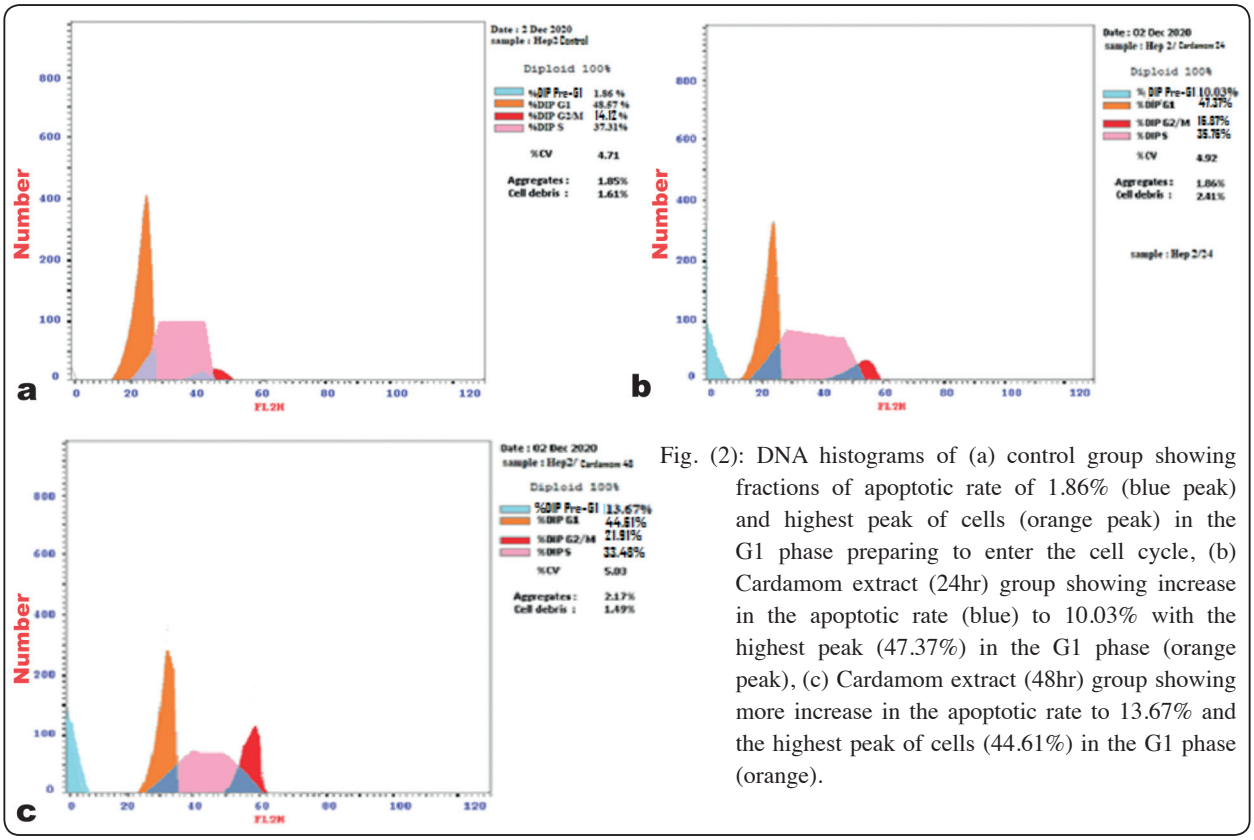


Fig. (2): DNA histograms of (a) control group showing fractions of apoptotic rate of 1.86% (blue peak) and highest peak of cells (orange peak) in the G1 phase preparing to enter the cell cycle, (b) Cardamom extract (24hr) group showing increase in the apoptotic rate (blue) to 10.03% with the highest peak (47.37%) in the G1 phase (orange peak), (c) Cardamom extract (48hr) group showing more increase in the apoptotic rate to 13.67% and the highest peak of cells (44.61%) in the G1 phase (orange).

Statistical Analysis

Regarding fold change of caspase-3 by RT-PCR: The highest mean value was recorded in DOX (48hr) group, followed by DOX (24hr) group, then Cardamom extract (48hr) group, then Cardamom extract (24hr) group, with the least value recorded in control group **Figure (3).**

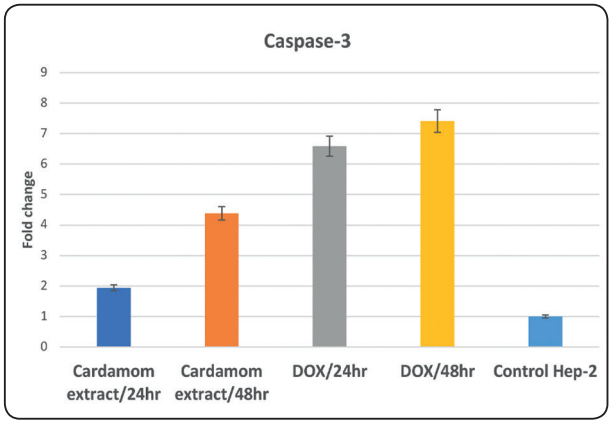


Fig. (3): Bar chart illustrating mean Caspase-3 fold change in different groups

DISCUSSION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with 890,000 new cases and 450,000 deaths in 2018. The incidence of HNSCC continues to rise and is anticipated to increase by 30% (that is, 1.08 million new cases annually) by 2030 [(11)]. Despite excellent functional and survival outcomes in patients with early-stage HNSCC, patients with advanced-stage disease continue to have poor survival [(12)]. Accordingly, HNSCC was the field of interest and the lesion of choice in the current study.

The complications and consequences of surgery, radiation therapy, and chemotherapy are associated with toxicity leading to some degree of late organ dysfunction that may be substantial whether a surgical or nonsurgical approach is taken [(1)]. Fortunately, some spices exerted their anticancer properties by inducing apoptosis, DNA damage causing G2-M arrest and inhibiting tumorigenesis, proliferation, invasion, metastasis and migration. The doses to achieve equivalent cancer control of radiation or chemotherapy drugs were lowered by combined treatment of spices, thus minimizing the adverse effects to normal tissues. The efficacy of existing chemotherapeutic agents and radiotherapy were enhanced, indicating that combined treatment is a potential therapeutic strategy for cancers. In a word, natural products are promising sources of adjuvant therapy of cancer. In the future, more anticancer bioactive components in natural products should be separated and identified, and the mechanisms of action should be further explored [(13)].

In the past few decades, the anti-tumor activity of Cardamom has been revealed in many cancer models as colon cancer [(14)], breast cancer [(7)], and non-melanoma skin cancer [(4)]. However, for the best of our knowledge, its effect on HNSCC has not been extensively studied in the available literature. Therefore, the possible anti-cancer effect of Cardamom extract on HNSCC was the point of interest in the current work.

In order to judge the beneficial action of Cardamom extract, it was very important to use a reference chemotherapeutic drug for comparing its results with those of Cardamom groups. Doxorubicin (DOX) was the drug of choice as it is a broad spectrum antitumor agent used in the treatment of solid tumors in adult and pediatric patients. It is one of the most effective anti-cancer drugs in several types of cancers [(15)].

In this study, multiple techniques were used, including Microculture Tetrazolium (MTT) assay, Flow Cytometry and Real time Polymerase chain reaction (RT-PCR). The findings of the present study revealed potential cytotoxic effect of Cardamom extract on Hep-2 cell line in a dose-dependent manner, as it succeeded to decrease the percentage of viable and proliferating cells with increasing its dose. On the other hand, it increased

the percentage of apoptotic cells and levels of caspase-3 with increasing time of treatment. This could be attributed to its anti-proliferative, anti-inflammatory and antioxidant activities.

The anticancer effect of Cardamom extract, which is revealed in this work, is in agreement with **Das et al., 2011**[4], **Jou et al. (2015)**[16], and **Vutakuri & Somara, 2018** [7].

Moreover, decreasing the IC_{50} value with increasing time of treatment from 24h to 72h time intervals indicates that the cytotoxic effect of Cardamom extract against Hep-2 cell line could be efficiently exerted with the same efficacy either by a high dose of Cardamom extract for limited time, or reduced Cardamom extract dose for prolonged time.

In the current work, cell cycle analysis results using Flow Cytometry in Cardamom extract and DOX treated groups revealed that there's a remarkable decrease in the percent of cells in the G0-G1 and S phases of cell cycle with increasing time of treatment which indicates that there is change in the DNA content of cells entering the cell cycle, leading to deregulated cell cycle and inhibition of cancer cell growth and proliferation. On the contrary, the results showed an increase in the percentage of cancer cells in G2-M phase, which indicates that Cardamom extract has caused irreparable DNA damage, which led to cell cycle arrest and accumulation of cells in this phase of cell cycle in preparation to apoptosis. This is in accordance with **Kong et al. (2019)** [17].

Moreover, the levels of caspase-3 were markedly elevated in Cardamom extract treated cells in comparison to control group and with increasing time of treatment from 24hr to 48hr, which in turn indicates increasing of apoptosis. This finding agrees with the results of **MURATA et al. (2013)** [18] and **Jou et al. (2015)** [16]. This supports the belief that Cardamom has exerted its anti-cancer effect via activation of caspase-3 which is a key molecule in regulating apoptosis.

CONCLUSION

The present study clearly demonstrated high success rate and promising Cardamom extract efficacy against HNSCC which was comparable to that of the well-known chemotherapeutic drug DOX. Accordingly, this study elucidates the possibility of using Cardamom extract as a complementary or adjuvant therapy for conventional cytotoxic therapies, having less toxicity and side effects.

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