

RESEARCH COMMUNICATION

Cytotoxic Potential of Indian Spices (Extracts) Against Esophageal Squamous Carcinoma Cells

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Abstract

Diet is one of the important factors in cancer etiology and prevention. The Indian diet is particularly interesting in its many unique dietary constituents, including spices like chili pepper, cloves, black pepper and black cumin, that have promise as chemopreventive agents. The objective of the present study was to compare the *in vitro* anticancer activities of aqueous and ethanolic extracts against the TE-13 (esophageal squamous cell carcinoma) cell line. All extracts showed cytotoxic activity but aqueous extracts were found to be more potent than alcoholic extracts. Morphological analysis, DAPI staining and DNA fragmentation assays showed maximum cell death and apoptotic cell demise (88%) to occur within 24 hours with an aqueous extract of chili pepper at 300µl/ml.

Keywords: Spices - esophageal cancer cells - TE-13 - cytotoxicity - DAPI - apoptosis

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Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common cancers in the world with extremely poor prognosis due to late presentation and rapid progression. It is eighth among the most common cancers worldwide and fifth most common cancer in developing countries (Aggrawal et al., 2003). The main reason for the development of esophageal cancer is the exposure to the environmental carcinogens. The etiology is supported by geographic and genetic factors (Murtaza et al., 2006). ESCC shows a great variation in its geographical distribution and the incidence rate is very high in certain parts of China, Iran, South Africa, Uruguay, France, Italy and in some regions of India (Stoner & Gupta, 2001). In India, the highest incidence of this cancer has been reported from Assam (the North-east region) where it is the second leading cancer in men and third leading cancer in women (Phukan et al., 2001) and Kashmir valley (Hussain et al., 2011). Despite advances in surgery and neo adjuvant therapy, 5- year survival rates for esophageal cancer are between 10 and 15%. Therefore, new approaches are required to target esophageal cancer cells to improve efficacy without toxicity.

An use of herbal remedies based on spices is one kind of complementary and alternative medicine (CAM) is documented in the population especially strong among cancer patients. (Boon et.al,2007; Xu et.al, 2006). The spice based herbal medicines and the constituents have been reported to inhibit the proliferation of cancer cells

directly.

Many emerging data in recent years have shown that dietary chemopreventive agents preferentially inhibit growth of cancer cells by targeting signaling molecules, such as caspases, that subsequently lead to the induction of apoptosis. (Khan et al., 2007). Some of these dietary agents include epigallocatechin-3-gallate (EGCG), found in green tea (Brusselmans et al., 2003) Curcumin in turmeric (Gao et al., 2005), genistein in soybeans (Su et al., 2006), lycopene in tomatoes (Liu et al., 2006), anthocyanins in pomegranates (Larrosa et al., 2006) and isothiocyanates in broccoli (Zhang et al., 2004). Some studies have also indicated that the herb and spice extracts could inhibit the proliferation of various cancer cell lines *in vitro* (Tang et al., 2007).

The ability of spices to show a broad range of activity has been attributed to its ability to affect multiple targets. However, how spices actually achieves this broad-spectrum activity is unknown. So far very few reports are available on herb and spices extracts in particular with reference to esophageal cancer. Herbs had antitumor effects on human esophageal cancer cells (ECCs) (Iizuka et al., 1998). Green tea also has been shown to reduce tumor multiplicity in the rat esophagus when administered in the drinking water (de Boer et al., 2004).

In the present investigation, human esophageal squamous carcinoma cells, TE-13 were employed to evaluate the anticancer properties of different spice extracts, and so far our knowledge this is the first time tested their efficacy against TE-13 cells.

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Materials and Methods

Cell culture

TE-13 (esophageal squamous cell carcinoma) cell line was procured from NCCS, Pune, and maintained in cell culture growth media, Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine serum (FBS) (Sigma chemicals, USA), and 1% penicillin/streptomycin (Sigma). Cells were cultured as adherent monolayer and incubated at $37 \pm 0.5^\circ\text{C}$ and 5% CO_2 and 95% humidity.

Preparation of spice extracts

Fresh spices (Chili Pepper, Clove, Black pepper and Black Cumin) were bought from main market in Ghaziabad, India. They were milled to fine powder with the aid of a clean electric blender. 50 g milled powder of each spice was soaked in 200 ml of distilled water to prepare the aqueous extract, and in 200 ml of ethanol to prepare the ethanolic extract. It was allowed to stand for 24 h after which it was filtered using a Whatman No. 1 filter paper and then the filtrate was evaporated to dryness with the help of rotary vacuum evaporator (Ijeh et al., 2005).

Treatment of cells

Cells were plated (1×10^4 /well) in 96-well plates in complete medium and cell viability was ascertained by trypan blue. After 24 hours, medium was removed and replaced by the fresh medium with and without various doses from 100-300 $\mu\text{l/ml}$ of water and ethanol extract of all the four spices. Another set of treatment also done to check the cytotoxic effect with synthetic form of active ingredients of corresponding spice in triplicate as described in legend of figures. The percentage of cell death was estimated by MTT assay.

MTT assay for cell viability

Cell viability was measured by MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide); Sigma). After 24 h treatment, MTT (5mg ml⁻¹ in phosphate buffered saline (PBS)) was added to each well and incubated for 4 h at 37°C in 95% air and 5% CO_2 . Viable cells had intact mitochondria and dehydrogenases present there which convert the tetrazolium salt to insoluble formazan violet crystals. Reduced MTT was dissolved in DMSO and measured spectro photometrically in a dual-beam microtiter plate reader (Biotek USA) at 570 with a 620-nm reference. Experiments were carried out in triplicate wells, repeated at least three times. Values are presented as the mean % cell viability \pm SD.

Apoptosis analysis

DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) staining was performed to see the morphology of the nuclei after treatment. The cells were grown in the 6-well plate. After reaching approximately 90% confluency, the cells were treated with different concentrations of extracts and were incubated for 24 hours. Cells were observed with inverted microscope after 24 hours to check on morphological changes, suffering from cell death. Then cells were washed twice with 1x PBS

and 0.01% of formaldehyde was added and mixed gently for 1 hour. After that the cells were again washed twice with PBS and DAPI was added and kept for 10 min in dark. Finally the cells were washed twice with PBS and suspended in 500 μl of fresh PBS and the morphology of nuclei was viewed under Fluorescence microscopy (Olympus 1x81 Inverted Research Microscope).

DNA fragmentation assay

After treatment, the cells were harvested by trypsinization and centrifuged at 1,000g for 5 min, washed once with PBS and the cell pellet was resuspended in a lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100. Cell lysate was left on ice for 30 min. DNA was extracted by adding an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (pH 8.0) and precipitated with 0.1 volume of 5 M sodium chloride and 2 volumes of 100% ethanol at -20°C overnight. The DNA sample was dissolved in TE buffer (10 mM Tris, pH 8.0 and 1 mM EDTA) and treated with 1 mg/ml RNase at 37°C for 2 h. DNA fragments were resolved by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining.

Statistical analysis

Data from three different set of experiments were analyzed and expressed as average % of cell viability + SD. Significant difference in cell death between control and treated cells values were also statistically analyzed using Student's t-test. A value of $p < 0.05$ was considered to be significant.

Results

Effect on cell proliferation

To verify the possible anti-proliferative effect of spice extracts as a first step toward the development of novel putative anticancer agents, we tested aqueous and ethanol extract of chili pepper, clove, black pepper & black cumin along with their known active ingredients Capsaicin, Eugenol, Piperine and Thymoquinone as standards respectively for check their capability to inhibit cell growth or viability on esophageal cancer cell line (TE-13). Cell proliferation assays were performed to test the possible cytotoxicity of different spice extracts. As it was shown in Figure 1 that at the dose of 300 $\mu\text{l/ml}$ water extract of chili pepper exhibits maximum cell growth inhibition where as black pepper extract exhibits least. Similarly ethanol extract of clove has maximum up to 55% cell death at 300 $\mu\text{l/ml}$ dose where as black pepper exhibits only 8% cell death at the same concentration. Interestingly it was observed that aqueous extracts of all 4 spices exhibits better cytotoxic activities in comparison to standards of their respective active ingredients.

Effect on morphology

In view of the above findings morphological analysis of aqueous extract treated cells was done and it was observed that cytotoxic effects of spices are associated with apoptosis. Cell shrinkage was shown in the treated cells, a major characteristics of nuclear fragmentation

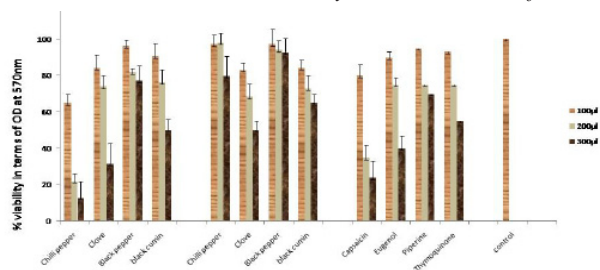


Figure 1. Comparative Analysis of Effect of Spice Extracts (Water, Alcohol) and Their Standards on TE-13 Cell Viability. Cell line (1×10^4 cells /well) was incubated with different concentrations ($100 \mu\text{l/ml}$, $200 \mu\text{l/ml}$ & $300 \mu\text{l/ml}$) of spice extracts for 24 hours. The cell viability was measured by MTT assay as described in material and methods. The data was obtained from independent triplicate experiments

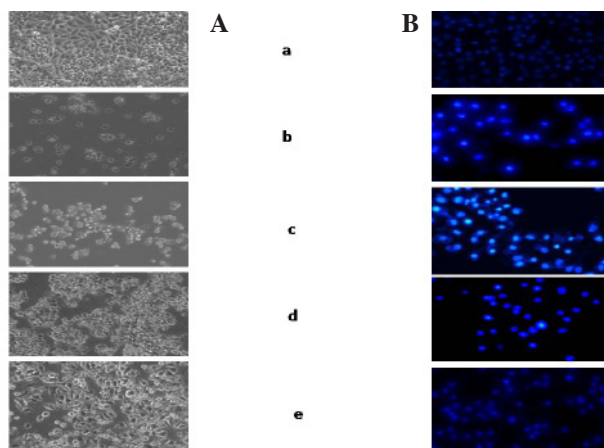


Figure 2. Morphological Appearance. (A) Phase contrast micrographs of TE-13 cells treated with $300 \mu\text{l/ml}$ aqueous extract of different spices for 24 hours. a) untreated cells b) treated with aqueous extract of chili pepper c) treated with aqueous extract of clove d) treated with aqueous extract of black pepper e) treated with aqueous extract of black cumin. (B) Fluorescence microscopic analysis of DAPI staining of TE-13 cells treated with $300 \mu\text{g/ml}$ aqueous extract of different spices for 24 hrs as described in material and methods.

due to which the dying of cells were taking place in comparison to untreated control cells. As shown in Figure 2(A) the maximum morphological changes were seen with red chili aqueous extract treated cells.

Effect on Apoptosis:

DAPI staining demonstrated that these extract induced DNA fragmentation

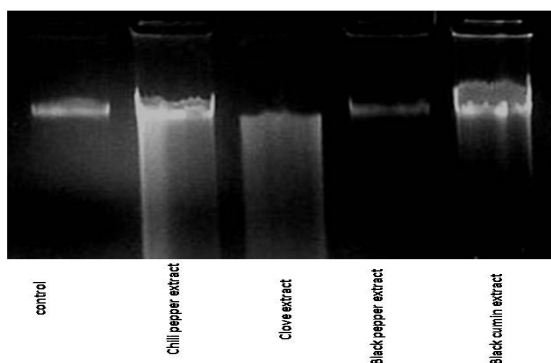


Figure 3. DNA Fragmentation Analysis of Treated Cells after 24 Hours

change in nuclear morphology. Compared to the typical round nuclei of the control, treated cells displayed condensed and fragmented nuclei. It was observed that level of apoptotic cell demise was maximum in aqueous extract of chili pepper. (Figure 2(B))

Further extent of apoptosis was confirmed by DNA fragmentation as shown in Figure 3 revealed the presence of fragmented nuclei characteristic of apoptosis after the treatment with aqueous extract of spices. In addition, a ladder like pattern of DNA fragmentation using agarose gel electrophoresis was observed at 24 hrs after the treatment.

Discussion

Modern day therapies like surgery, radiotherapy and chemotherapy have emerged with a very significant and effective role in the treatment of cancer but their added disadvantages, high costs and increasing resistance is a matter of concern. This leads to the discovery of an alternative and effective treatment, which has added benefits, less disadvantages, low costs, more significant effects without any toxic and harmful side effects. Exploring the nature and its natural compounds for the anti cancerous properties is one of the major and very recent research fields against the battle to overcome and treating cancer. So far sufficient literature is available in this regards for the treatment of different cancers except esophageal cancer therefore, present study gives new incite for the treatment of esophageal cancer.

Cancer is a leading cause of death worldwide. Various ethnic and racial groups are affected differently by overall cancer incidence and mortality. The reasons for differences in cancer incidence and survival are not entirely clear. However, diet plays an important role in cancer prevention and survival and may also be implicated in racial and ethnic disparities (Ferdowsian & Barnard, 2007).

In India incidence of esophageal cancer is moderately high and it is associated with diet and lifestyle. It is the second most common cancer in males and fourth most common cancer according to the combined cancer registry (Gajalamxmi et al., 2001).

The search for food and spices that can induce apoptosis in cancer cells has been a major study interest in the last decade. In India diet is one of the important factors in cancer etiology and prevention. Indians have one of the most interesting diets with many unique dietary constituents that have promise for cancer prevention. Many chemopreventive agents have been associated with anti proliferative and apoptotic effects on cancer cells because of their high antioxidant activity, targeting signaling molecules, and preventing or protecting cells from further damage or transformation into cancer cells. (Khan et al., 2007). A survey of the literature revealed that not much studies was done on the potential anticancer activity of spices except Curcumin (Ushida et al, 2000; Sullivan-Coyne et al, 2009) on esophageal cancer.

This is the first report in which we have investigated the cytotoxic activity of different spice extracts against esophageal cancer cell line. It is known that different spices might exhibit different sensitivities towards single

cell line; therefore it is necessary to take a panel of spices in the detection. Bearing this in mind, four different spices red chili, clove, black pepper and cumin were used in the present study along with their known active ingredients Capsaicin, Eugenol, Piperine and Thymoquinone as standards respectively. Interestingly it was observed that aqueous extracts of all four spices exhibits better cytotoxic activities in comparison to standards of their respective active ingredients.

This study first reported the effect of ethanolic and aqueous extract of spices in inhibiting squamous esophageal cancer cells. Our study is strongly supported by the findings of Kuete et al, 2011 in which they have investigated the cytotoxic potential of 34 different Cameroonian spice and herbal extracts on human pancreatic cancer cell line MiaPaCa-2, leukemia CCRF-CEM cells. Extract introduced apoptosis in cancer cells, which was determined by a loss of cell viability, nuclear shrinkage and apoptotic body formation as confirmed by the MTT assay, DAPI staining and internucleosomal DNA fragmentation. DNA damage is a common event in life following which, repair mechanisms and apoptosis will be activated to maintain genome integrity. The tumor-suppressor protein p53 provides an important link between DNA damage and apoptosis. DNA damage results in cell cycle arrest at checkpoints or at G1 or G2 stage to inhibit cell cycle progression and to induce apoptosis, thus protecting cells against further damage (Bortner et al., 1995).

The induction of apoptosis is known to be an efficient strategy for cancer therapy. Many Chinese herbal remedies such as *Ganoderma lucidum*, *Rubus coreanum*, *Paeoniae Radix* and *Phyllanthus urinaria* have been shown to trigger the apoptotic pathway in MCF-7 human breast cancer cells, HT-29 human colon cancer cells, HepG2 human hepatoma cells and Lewis lung carcinoma cells, respectively. (Hu et al., 2001; Lee et al., 2002; Huang et al., 2003; Kim et al., 2005). Our study also showed that spice extracts induced severe DNA damage in esophageal cancer cells. This suggested that spice extracts specifically inhibits cell growth and induces apoptotic cell death in esophageal cancerous cell line of human origin.

In conclusion, these spice extracts have an anti-proliferative effect in the esophageal cancerous cell line of human origin by inducing apoptosis. Therefore, our findings open up the possibility that natural compound found in these spices may be used to develop new treatment modality for esophageal cancer. However, further investigation is needed to determine the mechanism for its action in esophageal carcinoma.

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