RESEARCH COMMUNICATION

Comparative Anticancer Potential of Clove (*Syzygium aromaticum*) - an Indian Spice - Against Cancer Cell Lines of Various Anatomical Origin

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Abstract

Spices, active ingredients of Indian cooking, may play important roles in prevention and treatment of various cancers. The objective of the present study is to compare the in vitro anticancer activities of three different extracts of Clove (*Syzygium aromaticum L*), a commonly used spice and food flavouring agent, against different kinds of cancer cell lines of various anatomical derivations. Water, ethanol and oil extracts were screened for anti proliferative activity against HeLa (cervical cancer), MCF-7 (ER + ve) and MDA-MB-231 (ER – ve) breast cancer, DU-145 prostate cancer and TE-13 esophageal cancer cell lines, along with normal human peripheral blood lymphocytes. Inhibition of cell proliferation was assessed using MTT assay as a vital stain. In the examined five cancer cell lines, the extracts showed different patterns of cell growth inhibition activity, with the oil extract having maximal cytotoxic activity. Morphological analysis and DAPI staining showed cytotoxicity to be a result of cell disruption with subsequent membrane rupture. Maximum cell death and apoptotic cell demise occurred in TE-13 cells within 24 hours by clove oil at 300µl/ml with 80% cell death whereas DU-145 cells showed minimal cell death. At the same time, no significant cytotoxicity was found in human PBMC's at the same dose.

Keywords: Clove - eugenol - GLC - cytotoxicity - DAPI - apoptosis

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Introduction

Indian spices not only add aroma and taste to the food, but also possess certain medicinal values as well. Many Indian spices like turmeric, red chili, coriander, cumin, and mint have been proved to cure the diseases ranging from common cold and cough to cancerous tumors. Besides India, spices are also produced in several other parts of the world, but those produced on the Indian land are totally incomparable in attribute. Nowadays, these spices are getting much fame in the west as well.

Some of the spices are used as food, some are used for coloring and taste. The flavors are provided by the essential oils and oleoresins present in spices .Varieties of flavoring agents are used for their specific delicious taste. Some of these agents are rich sources of flavonoids which can block carcinogenesis. Some spices have anti-inflammatory and antioxidant activity with some added chemo preventive activity (Gordon, 1996; Shobana and Naidu, 2000; Ganguly, 2010). It has been observed that flavor enhancer spices can protect against a wide range of cancers, heart diseases and other chronic diseases (Craig, 1999).

Turmeric, ginger, garlic, capsicum, fenugreek, bay leaves, cinnamon have different active compounds with anticancer properties (Miler, 2001; Das, 2002; Hou et al., 2003; Lin et al., 2003; Kim et al., 2005; Mori et al., 2006; Su et al., 2006). Extracts of several commonly used Indian spices also have been shown to inhibit lipid peroxidation; in one study, relative antioxidant activities from highest to lowest were found in cloves, cinnamon, pepper, ginger, and garlic (Shobana and Naidu, 2000).

Among Indian spices turmeric is the most common ingredient. Curcumin, an active compound of turmeric was established as a potent anticancer compound after successful results in different *in vitro* and experiments (Johnson and Mukhtar, 2008). The compound acts as an anti proliferative agent by down regulating COX2, iNOS and cyclin D (Chun et al., 2003; Singh and Singh, 2009) and is now under phase I clinical study in patient with high risk or premalignant lesion (Cheng et al., 2001) .

Clove (*Syzygium aromaticum*) is one of the most commonly used spices in Indian kitchens. It has been shown to be a potent chemo preventive agent, used by the traditional Ayurvedic healers of India since ancient times to treat respiratory and digestive ailments (Banerjee et al., 2006). Essential oils present in the dried flower buds of clove are eugenol, caryophyllene, alpha-humulene, alpha-terpinyl acetate, eugenyl, methyl eugenol, actyl eugenol, naphthalene, chavicol, heptanone, sesquiterpenes,

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methyl salicylate pinene, vanillin (Duke and Cellier, 1993). The major chemical constituents of clove include sesquiterpenes, volatile oil (eugenol), caryophyllene, tannins and gum. Among different essential oils eugenol is the principle component, present in amount of 81.1%. Beside this trans-cryophyllene and isoeugenol are present in amount of 7% and 10.1% respectively (Zheng et al., 1992)

The main compound of clove is eugenol which is used as an antiseptic, antibacterial, analgesic agent in traditional medical practices. Now it is used in pharmaceutical and food products and in beverages as a flavoring agent. The therapeutic benefits of eugenol are well known. In recent times, it has been studied for a variety of promising biological properties. It has been reported to participate in photochemical reactions and to possess insecticidal, antioxidant and anti-inflammatory activities (Scott et al., 2009). Several studies have shown that clove has antiviral properties and have inhibitory effect on viruses like Herpes Simplex Virus (HSV) and hepatitis C virus (HCV) (Montes-Belmont and Carvajal, 1998; Shiraki , 1998).

In the view of antagonistic nature of different crude extracts of clove, we undertook the task to study the comparative anti-proliferative effect of these clove extracts on the panel of cancer cell lines from different anatomical site for the future therapeutic interventions.

Materials and Methods

Cell culture

HeLa (cervical cancer), MCF-7(ER +) and MDA-MB-231 (ER-) (breast cancer), DU-145(prostate cancer) and TE-13 (Esophageal cancer) cell lines were selected for this study. These cell lines were procured from NCCS, Pune and were 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± 0.5 ˚C and 5% CO_2 and 95% humidity.

Preparation of clove extracts

Fresh spice (Clove) was bought from main market in Ghaziabad, India. They were milled to fine powder with the aid of a clean electric blender. 50 g milled clove powder 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 the filtrate was evaporated to dryness with the help of rotary vacuum evaporator (Ijeh et al,2005). Clove oil extract was prepared by standard steam distillation process (Miles and Smiley, 2002). A stock of 100mg/ml was prepared for all 3 extracts and used for various assays.

Identification of active ingredient of Clove extract by GLC analysis

Eugenol, the active ingredient of clove oil is a volatile oil. Due to the vaporization, the purification of clove extract is achieved by GLC (Jirovetz et al., 2006). GLC (Gas Liquid Chromatography) is the best analytical tool for purification of volatile oil. The mobile phase is gas and the stationary phase is liquid in GLC. For GLC analysis 10µl/ml of clove oil extract was injected in GLC equipment and compared with commercially available eugenol as standard (Sigma).

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 (control) or supplemented with various doses of all three clove extracts in triplicate as described in legend of figures. The percentage of cell death was estimated by MTT assay.

MTT Assay for cell viability

After 24 h incubation, with different concentrations of clove extracts , MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml) 100μl/well was added, at appropriate time and incubated for 4 hours at 37°C. Viable cells had intact mitochondria and dehydrogenases present there which convert the tetrazolium salt to insoluble formazan violet crystals. The formazan crystals were dissolved in 200μl of dimethyl sulfoxide (DMSO). The absorbance was read at 570nm (A570) using an Absorbance Microplate Reader (BioTek, U.S.A).

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 clove at different concentrations 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 the 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 500µl of fresh PBS and the morphology of nuclei was viewed under fluorescence microscopy (Olympus 1x81 Inverted Research Microscope).

Statistical Analysis:

Data from three different set of experiments were analyzed and expressed as mean±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 and Discussion

Cancer is one of the major causes of death in all over the world. Recently, resistance to anticancer drug has been observed therefore more research is required to explore the anticancer potential of dietary and medicinal plant derived substances. Plants, vegetables, herbs, and spices used in

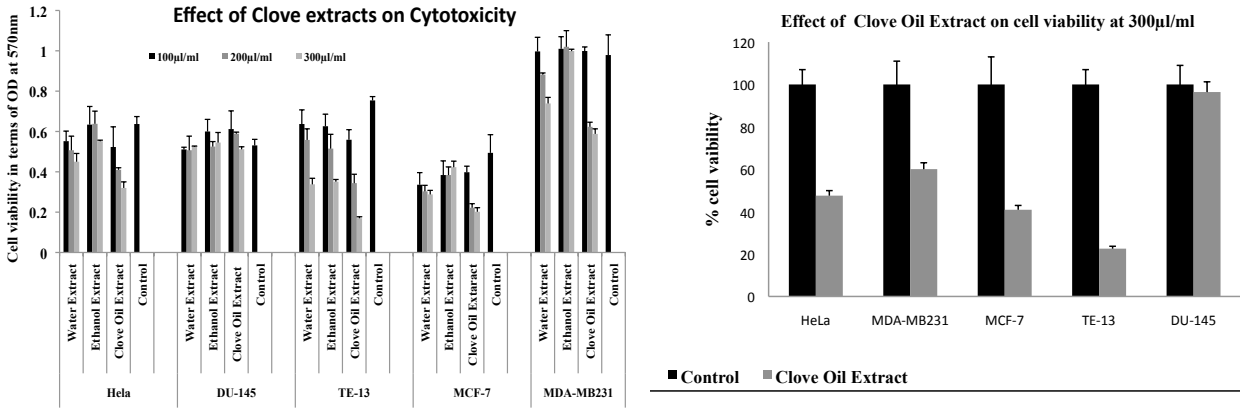

Figure 1. Comparative Analysis of Effects of Clove Extracts (water, alcohol & oil) on Cell Viability in 5 Different Lines. (a) Cell lines ($1x10^4$ cells/well) were incubated with different concentrations of different clove extracts for 24 hours. The cell viability was measured by MTT assay as described in material and methods. The data was obtained from independent triplicate experiments; (b) Effect of clove oil extract (300μl/ml) on the cell viability of 5 different cell lines in comparison to normal control cells

folk and traditional medicine have been accepted currently as one of the main sources of cancer chemo preventive drug. A large and increasing number of patients in the world use medicinal plants and herbs for health purposes. Therefore, scientific scrutiny of their therapeutic potential, biological properties and safety will be useful in making wise decisions about their use.

Research over the last decade has shown that several micronutrients in fruits and vegetables reduce cancer. The Active components of dietary phytochemical that most often appear to be protective against cancer are derived from spices and believed to suppress the inflammatory process that lead to transformation, hyper proliferation and initiation of carcinogenesis as well namely, angiogenesis and metastasis (Aggarwal and Shishodia, 2006).

The main finding of the present study is that clove has different capacity to cause cell death in the various cancerous cell lines of human origin. Clove (*Syzygium aromaticum*) is one of the most commonly used spice in Indian kitchens. It has been shown to be a potent chemopreventive agent, used by the traditional Ayurvedic healers of India since ancient times to treat respiratory and digestive ailments (Banerjee, 2006). The agent found in the clove extract is capable of killing cancer cells in the human body by proliferation-inhibiting and apoptosis-inducing (causing programmed cell death) effects.

To verify the possible anti-proliferative effect of clove extract as a first step toward the development of novel putative anticancer agents, we tested water, ethanol and oil extract of clove for their capability to inhibit cell growth or viability on a panel of human cancer cell lines of different anatomical origin. Cell proliferation assays were performed to test the possible cytotoxicity of different clove extracts. As a first screening, five cancerous cell lines from different body parts (see Methods section for cell lines details) were grown up to 24 hours in the presence or absence of different doses of clove extracts 100, 200 and 300μl/ml listed in Figure 1. As it was shown in Fig1 that esophageal cancer cell, TE-13 exhibits maximum cell growth inhibition for all three extracts where as in prostate cancer cells, DU-145 no significant cell growth inhibition was seen. On the other hand it was also observed that oil extract has maximum up to 80% cell death at 300μl/ml dose in comparison to other two extracts (Figure 1b). Similar to our observation, Parashar et al. (2006) reported that clove oil and eugenol have significant cytotoxic effect against human fibroblasts and endothelial cells .Our observations also correlated with another findings where they reported that eugenol has cytotoxic effect on human fibroblasts and Hep G2 hepatoma cell lines (Babich et al, 1993). No cell death was observed in normal human PBMC's by clove oil extract at higher concentration.

On the basis of these results we decided to further investigate the component of oil extract by GLC and its effect on cellular morphology and apoptosis. So for further work clove oil extract was chosen. GLC analysis was done to match the peak position between the crude oil extract and eugenol standard. After GLC analysis the highest peak was observed at same position as compared to standard eugenol. Thus, the GLC analysis of oil extract of clove indicated the presence of eugenol because of similar peak at same time was observed for both eugenol standard and clove oil analysis (Figure 2) therefore it may be concluded that the cytotoxic effect of oil extract found in different cancer cell lines is due to the presence of eugenol.

GLC analysis of clove oil extract shows two peaks is in same line (Figure 2). It may be mentioned that clove oil has two major compounds eugenol and β-caryophyllene,

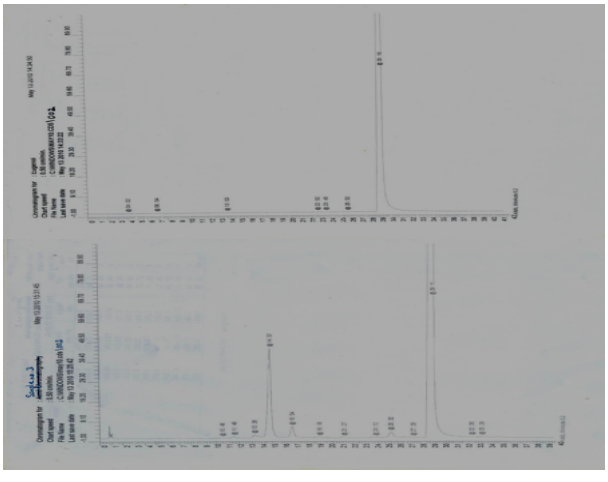

Figure 2. GLC Analysis of Clove oil Extract by Using Eugenol as the Standard

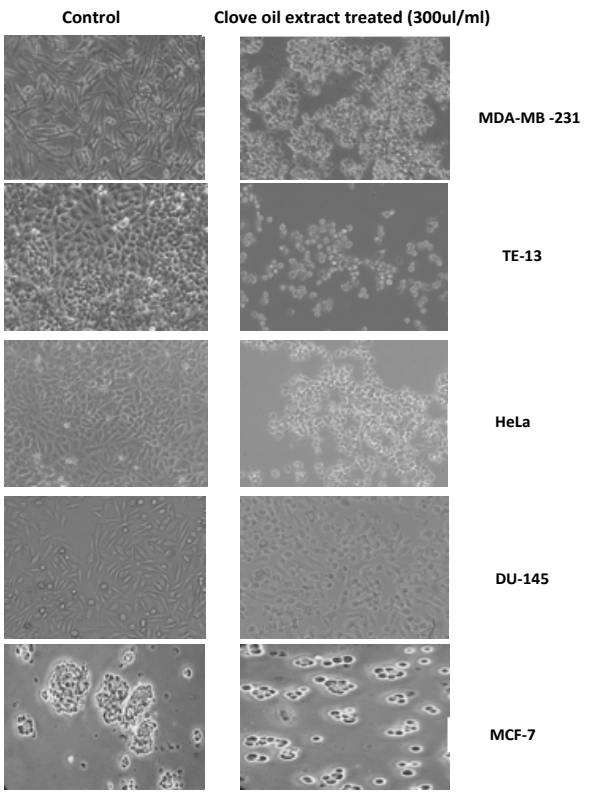

Figure 3. Phase Contrast Micrographs of Clove Oil Extract Treated Different Cell Lines

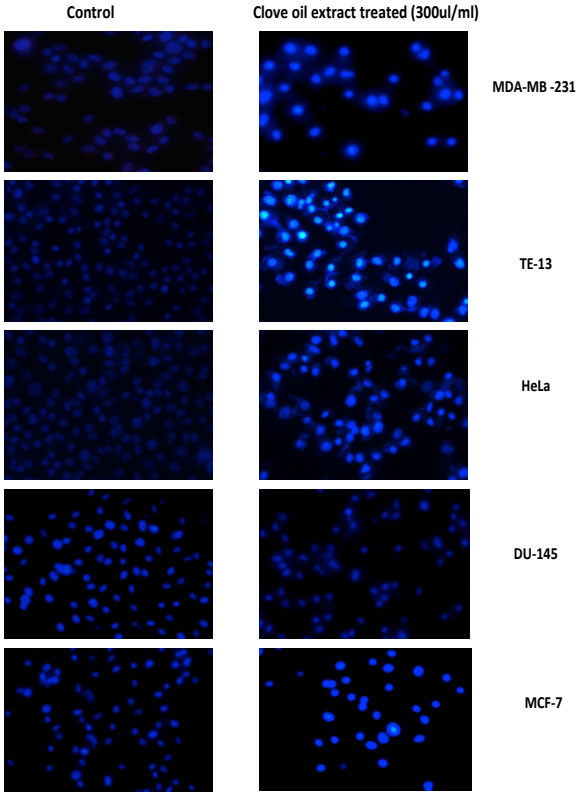

Figure 4. Fluorescence Microscopic Analysis of DAPI Staining of Different Cell Lines

which consists 79% and 13% of the oil respectively. Among these two β-caryophyllene had no cytotoxic effect whereas both clove oil and eugenol demonstrates cytotoxicity (Prashar et al., 2006).

Apoptosis is an important phenomenon in cancer chemotherapy, because anticancer drugs exert their antitumor effect against cancer cells by inducing apoptosis (Salomons et al,1999). Therefore, it is hypothesized that eugenol may exert its cytotoxic activity on different cancer cells by inducing apoptosis. In this study, we tested this hypothesis and found that the cytotoxic effect of eugenol is associated with apoptosis. In view of these finding we had performed DAPI staining and it was observed that cell shrinkage was shown in clove oil treated cells, a major characteristics of nuclear fragmentation due to which the dying of cells were taking place in comparison to untreated control cells. DAPI staining demonstrated that clove oil extract induced change in nuclear morphology. Compared to the typical round nuclei of the control, eugenol treated cells displayed condensed and fragmented nuclei. It was observed that level of apoptotic cell demise was maximum in TE13 (Figure 3 and 4). Further our findings correlated with the findings of Pisano et al. (2007) in their study they showed eugenol treated melanoma cells exhibits cytotoxic activity induced by apoptosis.

Therefore our findings open up the possibility that natural compound found in clove may be used to develop new treatment modality for esophageal cancer. As it is mentioned above that eugenol shows maximum cytotoxic effects against esophageal cancer cells this may be because after ingestion, sequence wise interaction between eugenol and cells in human body, esophageal cells are the first one in comparison to the other used cells. This finding again correlates with the recent published research by O'Sullivan-Coyne et al. (2009) were showed curcumin induced cell death in esophageal cancer.

In conclusion this work demonstrates that the eugenol present in clove oil extract is an effective cytotoxic agent for different type of cancer cells and it is endowed with apoptotic inducing capability. These results suggest that eugenol may constitute a potential antitumor compound against different kind of cancer cells depending up on their sensitivity towards it.

Acknowledgements

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