ORIGINAL RESEARCH

The Anticancer Effect of Allium Sativum & Foeniculum Vulgare Extracts in Combination On Oral Squamous Cell Carcinoma Cell Lines: An In Vitro Study

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Abstract

Introduction: The survival index of Oral squamous cell carcinoma/ Oral cancer/ OSCC has remained static over the past few decades. Besides, the low therapeutic efficiency & high toxicity effects of currently available conventional treatment options have recently highlighted the need for introducing primaeval ancient treatment modalities applying natural plant-based products.

Objectives: To assess the anticancer effect of Allium sativum & Foeniculum vulgare extracts, as a combination extract, on oral squamous cell carcinoma cell lines (KB and Cal 27 cell lines).

Methodology: Fresh samples of Allium sativum and Foeniculum vulgare were obtained from local markets of Mangalore, and KB & CAL 27 cell lines from the National Centre for Cell Sciences, Pune, India. Ethanolic extracts of Allium sativum & Foeniculum vulgare extracts as a combination mixture were prepared by amalgamating the plant powders with ethanol in a 1:5 ratio using the maceration method. KB & CAL 27 oral squamous cell carcinoma cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS & 1% antibiotic-antimycotic solution that was maintained at 37°C and 5% CO2 conditions in a humidified atmosphere. After three consecutive passages, the cytotoxicity of the compound was evaluated using an MTT assay. The data was subjected to statistical analysis.

Results: The mean and standard deviation of the combination extract of Allium sativum & Foeniculum vulgare for KB & CAL 27 at the highest concentration of 400µg/ml were 94.13 ± 0.68 & 93±1.06, respectively. The combination extract showed significant cytotoxic effects at higher concentrations against oral carcinoma cells (p-value < 0.05). The IC50 of combination extract for KB & CAL 27 cell lines was about 91.9 µg/mL & 117.6 µg/mL, respectively.

Conclusion: Allium sativum & Foeniculum vulgare extract proved to have high cytotoxic effect against human oral cancer cell lines, thereby inducing apoptosis & acting as a natural novel therapeutic agent in OSCC.

Key Words: Allium sativum, Foeniculum vulgare, Combination Extract, Oral Squamous Cell Carcinoma Cell Lines, Oral cancer, KB cell line, CAL 27 cell line

Introduction

The most common & challenging oral epithelial malignancies that has unveiled a static record of about 40% -50% of survival index from past many decades is Oral Squamous Cell Carcinoma /Oral Cancer/ OSCC. Being multifactorial in origin, this epithelial malignancy has documented high mortality index and poor quality of life (QoL), even after the currently available treatment options.[1,2] The limited success of conventional therapies i.e surgery, radiation, chemotherapy and immunotherapy have resulted in low therapeutic index and high toxicity with detrimental side effects, thereby indicating an imperative need for alternative therapies that are highly effective, safe, sustainable & less toxic with minimal side effects such as mild gastrointestinal discomfort and fatigue in detoxifying oral carcinogens in OSCC.[3,4] Natural plant-based derivatives and extracts, bestowed with phytochemicals and various medicinal benefits, have proved to be

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useful in treating different types of cancers, including OSCC, in recent years.[5] They provide a large source of natural antioxidants and bioactive compounds which could be useful in cancer management. Since they exert an antioxidant property, the mode of mechanism lies in decreasing the free reactive radicals (ROS) and the DNA repair mechanism, thereby acting as cancer protective and cancer preventive role in oral cancer.[6,7] Discovering their role in assessing the anticancer effect with the main bioactive component involved can open new avenues in the field of oral oncology. Additionally, to study the behavior of the malignant cells in a controlled environment, application of human oral cancer cell lines can aid to study specific cell types that display key characteristics in a defined population of cell by retaining their functions for an extended period of time.

Garlic (Allium sativum) & Fennel (Foeniculum vulgare) have been used for thousands of years as traditional herbs for medicinal purposes. Key anticancer compounds in garlic include Allicin, diallyl sulfide, diallyl disulfide and S-allyl cysteine. These are well-known for the induction of apoptosis, suppression of cell proliferation and angiogenesis and modulate the metabolism of carcinogens.[8] Anethole, a phenylpropanoid compound found in fennel has been shown to inhibit cancer cell proliferation, induce apoptosis, and modulate inflammatory and oxidative stress signaling pathways. Recently, both have gained attention in the medical field as herbal medicine & therapeutic agents for treating various diseases and conditions such as diabetes, atherosclerosis, hypertension, microbial infections, cardiovascular diseases, cerebrovascular diseases, dementia, Alzheimer's disease, allergies & even cancer.[9-12] Bestowed with phytochemicals, they are highly efficient, accessible, sustainable & safe, thereby exerting a wide range of antioxidant properties. Various meta-analytical studies[13-21] have documented the cancer preventive properties of both plant extracts in OSCC individually, however, no studies have yet evaluated the efficiency of both plant products as combination mixtures on cancer cell lines. Based on this platitude, we aimed to assess the anticancer effect of Allium sativum & Foeniculum vulgare as a combination extract on oral squamous cell carcinoma cell lines (KB and CAL 27 cell lines).

Methodology

This is an in vitro study that was initiated after due approval from the Institutional Ethical committee bearing the Protocol no YEC-1/2022/239. After a thorough literature review, two medicinal plants namely Allium sativum and Foeniculum vulgare were chosen for the present study. The conceptual framework design of the study was based on the identification of the research gap & research question so as to assess the anticancer effect of Allium sativum and Foeniculum vulgare as combination extract in OSCC cell lines namely KB & CAL 27. We applied the null hypothesis as "Allium sativum and Foeniculum vulgare extracts have no anticancer effect on oral squamous cell carcinoma cell lines when used in combination". The conformity of the set hypothesis was tested at 0.05 level of significance. The inclusion criteria were samples of all variants of OSCC. The exclusion criteria were oral potentially malignant disorders or any other oral pathologies not associated with OSCC.

Fresh Samples of Allium sativum & Foeniculum vulgare were obtained from the local markets of Mangalore. The samples were then authenticated by a botanist, and the voucher samples were deposited in the department. They were shade-dried and powdered using a blender. Ethanolic extracts were prepared by mixing both the plant powders with ethanol in

Table 1: Depicts Cytotoxicity of Combination Garlic & Fennel - KB cells group at various concentrations:

Concentration (µg/mL)	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Mean	Standard deviation
12.5	20.15571	18.94464	19.20415	20.24221	19.72318	17.47405	19.29	1.03
25	32.87197	32.35294	36.76471	33.3045	33.56401	32.35294	33.54	1.66
50	51.47059	50.60554	50.08651	51.47059	52.33564	51.90311	51.31	0.83
100	61.76471	60.98616	59.25606	60.81315	62.37024	64.27336	61.58	1.69
200	81.05536	81.57439	80.27682	79.84429	81.40138	80.70934	80.81	0.67
400	94.37716	93.3391	94.63668	94.29066	94.89619	93.2526	94.13	0.68

Table 2: Depicts Cytotoxicity of Combination Garlic & Fennel - CAL27 cells group at various concentrations:

Concentration (µg/mL)	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Mean	Standard deviation
12.5	3.148751	3.474484	4.234528	3.69164	2.823018	2.171553	3.26	0.72
25	13.35505	15.41802	16.17807	15.20087	15.85233	11.1835	14.53	1.91
50	45.38545	42.88817	41.15092	39.63084	39.95657	45.92834	42.49	2.71
100	77.09012	77.74159	77.41585	76.43865	73.83279	77.95874	76.75	1.52
200	87.62215	86.4278	85.55917	88.16504	87.62215	88.8165	87.37	1.18
400	94.13681	93.15961	93.81107	92.94245	91.09663	92.83388	93	1.06

1:5 ratio using the maceration method. Briefly, 20 g of the plant powder was mixed with 100 mL of ethanol and macerated on a rotary shaker for 24 h, maintained at 120 rpm. The extracts were filtered, concentrated using a rotary evaporator and stored at 4°C until further analysis. OSCC cell line (KB Cells and CAL 27) were used in the present study. The cell lines were procured from the National Centre for Cell Sciences, Pune, India. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum and 1% antibiotic- antimycotic solution (Gibco, Thermo Scientific, Germany). They were maintained at 37°C and 5% CO_2 conditions in a humidified atmosphere. Cells were used for the experiments after three consecutive passages. Cytotoxicity of the combination extract was assessed on the KB and CAL27 cells using MTT assay (Vybrant® MTT Cell Proliferation Assay Kit, cat no: M6494 (Thermo Fisher, Germany). Cells were seeded at a density of 5000 cells/well in 96-well microtiter plates and incubated at 37ºC under 5% CO_2 in a humidified atmosphere for 24 h. Allium sativum & Foeniculum vulgare as combination extract were added to the cells at different concentrations of 12.5, 25, 50, 100, 200 and 400 µg/mL and incubated further for 48 h. 100 µL of MTT solution (1mg/mL) (Invitrogen, Thermo Scientific, Germany) was added to the wells and incubated for 4 h. Formazan crystals were solubilized in DMSO and absorbance was recorded at 570 nm using a multimode microplate reader (FluoSTAR Omega, BMG Labtech). The cytotoxic effect of serial doses of combination extracts at each test was done by XY curve and subjected to linear regression analysis. Statistical significance between the untreated control group and the treated group was determined using one-way ANOVA. $p<0.05$ was considered significant.

Results

In the present study, the anti-cancer effect of Allium sativum & Foeniculum vulgare as combination extracts on oral squamous cell carcinoma cell lines i.e KB and CAL 27 cell lines, were assessed and analysed using MTT assay. The mean and the standard deviation values were calculated. The values obtained with serial concentrations for KB & CAL 27 cell lines are depicted in Tables 1 & 2, respectively. The mean cytotoxicity percentage and standard deviation of ethanolic extract as combination extract for KB & CAL 27 cells at 400µg/mL was

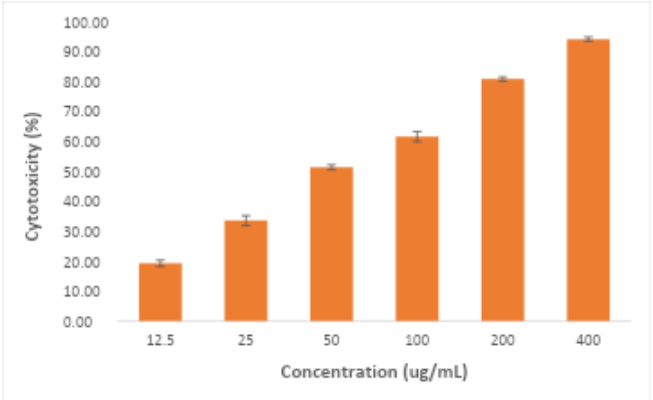

Fig. 1: Depicts Cytotoxicity of Combination Garlic & Fennel - KB cells group at various concentrations:

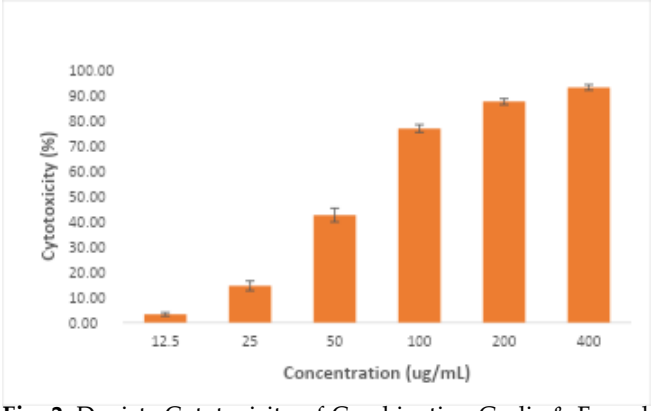

Fig. 2: Depicts Cytotoxicity of Combination Garlic & Fennel - CAL27 cells group at various concentrations:

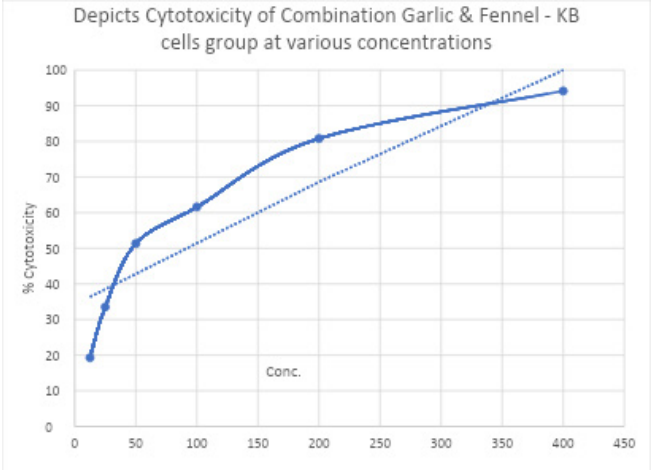

Fig. 3 Graphical representation of IC 50 value for Combination Garlic & Fennel - KB cells group at various concentrations:
*IC50 Value = 91.84302326 = 91.9 µg/mL

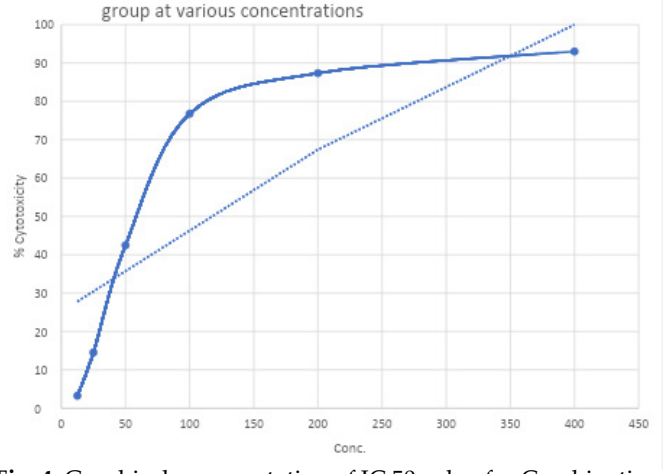

Fig. 4: Graphical representation of IC 50 value for Combination Garlic & Fennel – CAL 27 cells group at various concentrations:
*IC50 Value = 117.5378788 = 117.6 µg/mL

94.13 ± 0.68 & 93±1.06 respectively (Figure 1 & 2). The combination extract showed significant cytotoxic effects at higher concentrations against oral carcinoma cells (p-value < 0.05). The IC_{50} of the combination extract for KB & CAL 27 cell lines was about 91.9 µg/mL & 117.6 µg/mL, respectively (Figures 3 & 4). The difference was considered significant with p< 0.05. The lowest concentration of the extract used (12.5 µg/mL) exerted a percentage cytotoxicity of 19.29 ± 1.03, while the effect was enhanced with an increase in the concentration of the extract on the KB cells. The extract exerted cytotoxicity of 33.54 ± 1.66, 51.31 ± 0.83, 61.58 ± 1.69, 80.81 ± 0.67 and 94.13 ± 0.68 at concentrations of 25, 50, 100, 200 and 400 µg/mL, respectively. A similar trend was observed in case of CAL 27 cells. The lowest concentration of the extract used (12.5 µg/mL) exerted a percentage cytotoxicity of 3.26 ± 0.72, while the effect was enhanced with an increase in the concentration of the extract on the KB cells. The extract exerted cytotoxicity of 14.53 ± 1.91, 42.49± 2.71, 76.75 ± 1.52, 87.37 ± 1.18 and 93 ± 1.06 at concentrations of 25, 50, 100, 200 and 400 µg/mL, respectively.

Discussion

In the present study, we evaluated the anticancer effect of Allium sativum and Foeniculum vulgare extracts on KB & CAL 27 cell lines, in combination, in oral squamous cell carcinoma (OSCC). A combination of ethanolic extracts was used to check the cytotoxicity against oral squamous cell carcinoma cell lines i.e KB & CAL 27. Six different concentrations of ethanolic extract of Allium sativum, Foeniculum vulgare and a combination was chosen to test the cytotoxic efficacy. An astounding positive response was observed and the results also suggested that the surviving cancer cell lines count was significantly lowered when treated with combination extracts imparting high cytotoxic effect and possibly resulting in apoptosis of cancer cells. In case of Allium sativum, the main active biological ingredient were water soluble & oil soluble organosulfur compounds (OSCs) such as S-Allylcysteine (SAC), metabolites allyl mercaptan (AM), S-allyl mercaptocysteine (SAMC) & Allyl methyl sulfide (AMS), DAS (Diallyl sulfide), DADS (diallyl disulphide), DATS (diallyl trisulfides), DATTS (diallyl tetrasulfides), s-allylcysteine, vinyldithiines, allylpropyl disulfide, ajoene & allicin whereas in case of Foeniculum vulgare, the main active biological ingredients were 3-O-Caffeoylquinic acid, quercetin 3-rutinoside, anethole, trans-anethole, estragole (methyl chavicol), α-pinene, camphene, limonene, a-phellandrene & fenchone.[22-26]

Few evidence-based studies[13-21] were conducted in the past to assess the role of Allium sativum and Foeniculum vulgare extracts individually. The present study is the first of its kind to apply a combination of plant extracts on OSCC cell lines. The derived inferences from these previous studies concluded that plant-based extracts and their phytochemicals can positively exert anticarcinogenic effects through DNA damage& apoptosis, involving several modes of action at the cellular levels in carcinogenetic cascade.

Mapping of these studies[13-21]] showed the downregulation of cyclins D1, CDK-4, c-Myc proteins, P450 1A1, 1A2, 2B1 or 3A4 enzyme activity, scavenging free radicals, inhibiting protein folding in the endoplasmic reticulum & suppressing mutagenesis, detoxification by binding to their specific phytochemicals, differentiation & angiogenesis, preventing proliferation of malignant cells, enhancing apoptotic activity & evasion of immunosurveillance as the mechanism to inhibit cancer cell growth and apoptosis. The role of DATS in Allium sativum and the role of anethol in Foeniculum vulgare were shown to modulate cellular signalling pathways that alter the cellular defence mechanisms to protect normal cells from reactive oxygen species (ROS) and induce apoptosis in cancer cells. Our study was in accordance to these studies and additionally proved that combination extracts can be more effective in inducing apoptosis in OSCC. The bioactive compounds diallyl trisulfide (DATS) from Allium sativum and anethole from Foeniculum vulgare have been shown to modulate key cellular signaling pathways associated with cancer progression and oxidative stress. DATS exerts its anticancer effects by activating pro-apoptotic proteins such as p53, Bax, and caspase-3, while simultaneously suppressing anti-apoptotic proteins like Bcl-2. It also inhibits NF-κB signaling and induces mitochondria-mediated apoptosis. Additionally, DATS enhances the antioxidant defense system, helping protect normal cells from reactive oxygen species (ROS) damage.

Similarly, anethole has demonstrated the ability to modulate oxidative stress and apoptosis by inhibiting the PI3K/Akt and MAPK pathways, inducing cell cycle arrest, and promoting the activation of caspase-9 and caspase-3. These mechanisms not only suppress tumor cell proliferation but also confer cytoprotective effects on normal cells by enhancing redox balance.

Our findings align with the previous studies[22–26] and further reveal that a combination of garlic and fennel extracts leads to a synergistic effect, resulting in significantly greater apoptotic activity in oral squamous cell carcinoma (OSCC) cells than either extract alone. This enhanced efficacy likely stems from the complementary and multi-targeted actions of DATS and anethole in modulating oxidative stress responses and apoptotic pathways.

Limitations & Future scope

This study documented few limitations. The anticancer efficiency of Allium sativum & Foeniculum vulgare as combination extract on OSCC cell lines was assessed on oral squamous carcinoma cells, resulting in the generalizability of study findings. Future studies on animals, as well as clinical studies and cause and effect relationship between the predictors (exposure & outcome) can be analysed. However, this study can act as a pilot study on the potential uses of combination extracts, especially in terms of developing novel plant-based bioactive drugs in OSCC for the benefit of mankind.

Conclusion

Combination extracts of Allium sativum & Foeniculum vulgare proved to have high cytotoxic effect against human oral cancer cell lines (KB cell lines and CAL 27 cell lines), thereby inducing apoptosis & acting as a natural novel therapeutic agent in OSCC cell lines. Hence, this combination can be considered as a good anticancer agent against OSCC, thereby reducing toxicity to patients with cancer.

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