ORIGINAL RESEARCH

Cytotoxic effect of anise seed *(Pimpinella anisum)* extract on KB cell line – a comparative study with CISPLATIN

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Abstract

Introduction: Numerous studies have stated an association of spice consumption with lowered incidence of oral squamous cell carcinoma and other potentially malignant disorders of oral cavity. Anise seeds when consumed have the potential medicinal benefits including anticancer, anti-helminthic, hepato-protective and antioxidant activities. In this study we examined the anti-cancer activity of Anise seed extract (aqueous extract) on KB cancer cell lines.

Aims: a) To determine the cytotoxic activity of Anise seed extract on KB cell line b) To determine the cytotoxic activity of CISPLATIN on KB cell line c) To compare the cytotoxic activity of Anise seed extract and CISPLATIN on KB cell line.

Methods and Material: The study was conducted in Biogenix Research Centre, Thiruvananthapuram. The KB cancer cell lines were procured from NCCS, Pune. Anise seeds were purchased, dried and powdered using mortar and pestle. An extract was prepared in ascending concentrations and added to KB cell line in vitro under standardized environment. An extract of CISPLATIN at the same concentrations were prepared and added to KB cell line separately. Cytotoxic activity of the anise seed extract and CISPLATIN extract were assessed using MTT assay and cells were observed under an inverted phase contrast microscope, for any detectable changes in the cell morphology.

Results: The results obtained showed that with ascending concentrations of anise seed extract there was significant cytotoxic changes observed with p value significant and obtained at 0.0052.

Conclusion: Since anise seeds have been known to have a great potential as a chemotherapeutic agent, further focused studies of its anticancer properties and isolation of compounds from *Pimpinella anisum* are necessary to prove its worth in the cancer therapy

Key words: KB cell lines, anticancer activity, MTT assay

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Introduction

Cancer can affect almost any part of the body and is presently a major cause of morbidity and mortality in India. The most frequent cancers reported in India have been known to occur in breast, cervical, oral cavity and lung constituting majority of cancer population.[1]

Recent studies have predicted that occurrence of cancer in India is bound to double in the coming 10-20 years (Cancer in India, August 05/2019 report). As per the last Indian Council of Medical Research (ICMR) data update India had 14 lakh cancer patients in 2016 which is expected to increase[1].

Oral squamous cell carcinoma (OSCC) is a major public health problem in the Indian subcontinent, where it ranks among the top three types of cancer in the country[2]. The low income groups in India are affected most due to a wide exposure to risk factors such as tobacco chewing and insufficient exposure to new diagnostic aids, resulting in a delay in reporting of OSCC[3]. Ideally OSCC are treated with surgery followed by chemotherapy and or radiotherapy. CISPLATIN is the most commonly used chemotherapeutic agent used to treat a various types of carcinomas due to its broader efficacy[4]. The mechanism of action of CISPLATIN has been associated with ability to crosslink with the purine bases on the DNA to form DNA adducts, leading to cell cycle arrest at S, G1 or G2-M preventing repair of the DNA leading to DNA damage and subsequently induces apoptosis within cancer cell[5].CISPLATIN is administered intravenously as short-term infusion in normal saline for treatment of solid and haematological malignancies[6]. A number of side effects have been reported with the use of CISPLATIN like nephrotoxicity, neurotoxicity, ototoxicity, electrolyte imbalance etc that usually limit its use[7].

Even though CISPLATIN is a potent cytotoxic chemotherapeutic drug it has been noted that relapse of carcinomas and resis-

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tance to chemotherapy is commonly noted in many patients treated with CISPLATIN thus reducing its clinical efficacy.[8] Resistance to CISPLATIN can be an intrinsic phenomenon or it can be acquired due to chronic drug exposure. The main reasons attributed to drug resistance are intracellular accumulation of the drug, increased repair of the damaged DNA and cytosolic inactivation of CISPLATIN[8,9].

Resistance to the drug coupled with its adverse effects prompted researchers to start looking for alternatives in the form of natural bioactive compounds that could be incorporated to chemotherapeutic medicines to increase their efficacy and simultaneously lower their toxicity.

Several spices have been known to be potential sources for prevention and treatment of cancers, such as *Curcuma longa* (tumeric), *Zingiber officinale* (ginger), *Allium sativum* (garlic), *Piper nigrum* (black pepper) and *Capsicum annum* (chili pepper) induce apoptosis, inhibiting proliferation, migration and invasion of tumors, and sensitizing tumors to radiotherapy and chemotherapy[10].

Pimpinella anisum L. is an annual herb and a grassy plant with white flowers and small green to yellow seeds, which grows in warmer regions like India. *Pimpinella anisum* seeds are used in the treatment of variety of ailments like tooth ache, diuretic, digestive problems, carminative, expectorant, antispasmodic, antiseptic, flavoring agent in food industry etc.[11] Efficacy of anticancer properties of Anise seed extract is limited to human prostate cancer cell line PC-3, gastric cancer cell line AGS.[12,13]

However, to the best of our knowledge its anticancer property on oral squamous cell carcinoma has not been explored. Human Cancer cell lines are widely used to test emerging new therapeutic strategy in order to improve the efficacy of cancer treatment.

KB Cell line: The KB cell line was established in 1955 by Harry Eagle, reportedly from an epidermoid carcinoma (now known as squamous cell carcinoma, SCC), from the larynx of a male donor. However, in 1966 Gartler showed that they infact corresponded to Hela cell lines.[14] KB cells have been reported to contain human papilloma virus 18 (HPV-18) sequences. The KB cells are positive for keratin by immunoperoxidase staining[15].

Methyl Tetrazolium Assay (MTT) are cell-based assays often used for screening collections of compounds to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death. This type of cell-based assay is used to know the number of viable cells at the end of the experiment which are detected by tetrazolium compounds like methyl tetrazolium which readily penetrate viable cells[16].

The MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS)[17].

Materials and Methods

Cell culture

We used KB cell lines in our current study which was procured from National Centre for Cell Sciences (NCCS) Pune, India as per the guidelines. It was maintained in Dulbecos modified Eagles medium (DMEM) (Gibco, Invitrogen). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37°C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

Preparation of the extract

This current study was conducted at a private lab Biogenics Research centre in Trivandrum.

The extracts were prepared as follows

Extract 1: store bought *Pimpinella anisum* seeds were dried, powdered and processed for extraction using cold extraction method. The seeds were purchased, ground to a fine powder and mixed with ethyl acetate which was then kept in the open for partial evaporation in an extraction plate and extracted using Soxhlet apparatus. The fresh extract was then 5 times serially diluted by 2 fold dilution in 500ml of 5% DMEM to obtain the necessary five ascending concentrations of (6.25, 12.5, 25, 50, 100µg/mL) solutions. The sample solution was then filtered through 0.2 µm Millipore syringe filter to ensure the sterility.

Extract 2: CISPLATIN extract: 1mg of sample was weighed and dissolved in 1mL DMEM using a cyclomixer. The sample solution was filtered through 0.22 µm Millipore syringe filter (Millex GP) to ensure the sterility.

Cell Treatment Procedure

Two days old confluent monolayer of cultured cells were trypsinized and the cells were suspended in 10% growth medium, seeded in 96 well tissue culture plate and incubated at 37°C in a humidified 5% CO_2 incubator. After 24 hours the growth medium was removed, cells were treated with *Pimpinella anisum* extract of increasing concentrations (6.25,12.5,25,50,100µg/ml) in respective wells and incubated for 24 hours. Similar procedure was repeated with CISPLATIN extract which was of the same concentration of anise seed extract (6.25,12.5,25,50,100µg/ml).

Cytotoxicity Assay by Direct Microscopic observation:

Cells treated with extracts in the entire plate were observed at a regular interval of 24 hours; up to 72 hours under an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observations were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.[18]

Cytotoxicity Assay by MTT Assay Method:

15 mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS (phosphate buffered saline) until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37°C in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO) was added and the wells were mixed gently by pipeting up and down in order to solubilise the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (Laura B. Talarico et al, 2004).

The cytotoxic property of extract 1 and 2 were assessed as percentage of growth inhibition and calculated using the formula.

$$\text{Percentage viability} = \frac{\text{Mean OD Samples X 100}}{\text{Mean OD of control group}}$$

The LC_{50} value was calculated using ED50 PLUS V1.0 Software.

Results

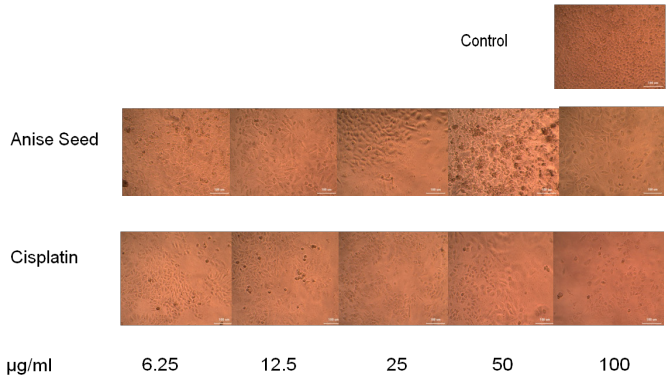
In the present study, the KB cell lines were incubated in ascending grades (6.25,12.5,25,50,100 µg/mL) of anise seed extracts and the cytotoxic activity of the extracts were assessed for cellular morphological changes under a microscope and measured using MTT assay. The LC_{50} value was calculated based on the percentage of the viable cells.

The cytotoxicity of the extracts were assessed

- based on changes in cellular morphology of incubated KB cells
- Using MTT assay which measures viability of cells

Changes in cellular morphology were visualized under inverted phase contrast microscope.

The cellular changes which were indicators of cytotoxicity are rounding or shrinking of cells, granulation and cytoplasmic vacuolization. We noted that cytotoxic changes in the incubated KB cells observed under microscope were directly proportional to increase in concentration of anise seed extract as well as CISPLATIN extract.



Apart from changes in cellular morphology the cytotoxic changes were also assessed using MTT assay.

It is based on the principle that MTT is positively charged and readily penetrates eukaryotic cells and thus can be done only on metabolically active cells. Viable cells convert MTT into formazan crystals which give a purple color and the deepening of intensity of the color obtained was directly proportional to the amount of viable cells.

We noted that the viability of the cells decreased along with increase in the concentration of anise seed extract i.e, the lowest concentration of anise seed extract 6.25 the viablility of cells were 92.62% whereas anise seed extract concentration of 100 showed 46.49% viable cells.

Sample code: ANISE SEED

Sample Concentration (µg/ml)	OD value I	OD value II	OD value III	Average OD	Percentage Viability
Control	1.4418	1.4635	1.4582	1.4545	100.00
6.25	1.4394	1.4250	1.4075	1.4240	97.90
12.5	1.3520	1.3617	1.3303	1.3480	92.68
25	0.9798	0.9967	0.9873	0.9879	67.92
50	0.7569	0.7315	0.7226	0.7370	50.67
100	0.4971	0.4491	0.4583	0.4682	32.19

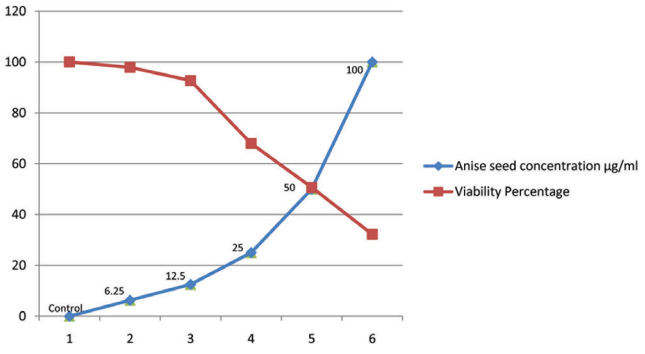
Similarly we noted that as the concentration of CISPLATIN extract increased the viability of the cells decreased i.e., lowest concentration 6.25 of CISPLATIN extract showed 43.99% of viable cells and 12.96% of viable cells at 100 concentration.

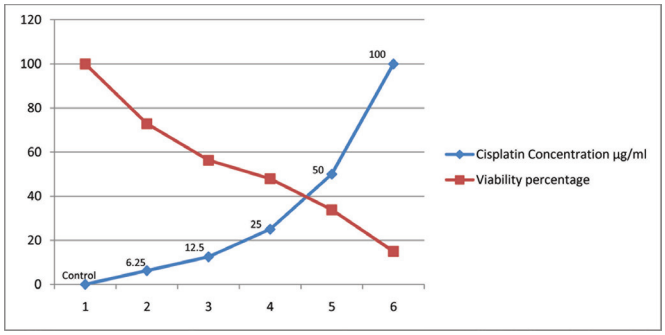
Sample code: CISPLATIN

Sample Concentration µg/ml	OD value I	OD value II	OD value III	Average OD	Viability percentage
Control	1.4418	1.4635	1.4582	1.4545	100.00
6.25	1.0815	1.0864	1.0121	1.0600	72.88
12.5	0.8117	0.8534	0.7915	0.8189	56.30
25	0.6895	0.6981	0.7020	0.6965	47.89
50	0.4922	0.4926	0.4917	0.4922	33.84
100	0.2089	0.2263	0.2174	0.2175	14.96

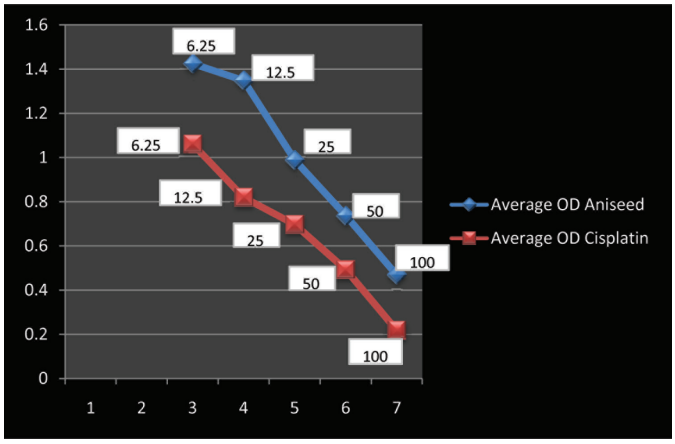
The viability of the cells were expressed by formation of purple formazan crystals the intensity of which was measured as optical density OD values. The OD value were recorded for control as well as samples of increasing concentration of anise seed and CISPLATIN extract.

Sample Concentration (µg/ml)	Viability percentage Anise seed	Viability percentage CISPLATIN
6.25	97.90	72.88
12.5	92.68	56.30
25	67.92	47.89
50	50.67	33.84
100	32.19	14.96





In our study we observed that the average OD value decreased with increase in concentration of both anise seed extract and CISPLATIN extract which in turn represented a decrease in percentage of viable cells.



The OD values obtained were entered into excel sheets and an average OD value was calculated and thereafter percentage of viable cells were estimated.

Based on the % of viability of cells LC_{50} value was calculated.

LC_{50} is the lethal concentration required to kill 50% of the population cells. In this current study the

The LC_{50} for Anise seed extract is 48.0705 µg/mL

LC_{50} for CISPLATIN extract is 30.0058µg/mL

The values were then statistically analyzed.

Discussion

Oral cancer is one of the 10 most common cancers in the world, with a delayed clinical detection, poor prognosis, without specific biomarkers for the disease and expensive therapeutic alternatives[19]. In worldwide reports, cancers of all regions of the oral cavity and pharynx are grouped and collectively represent the sixth most common cancer in the world[20].

Among the approaches to the treatment of OSCC such as surgery, radiation therapy (external beam radiotherapy and/or brachytherapy), and coadjuvant therapy (chemotherapy with agents such as CISPLATIN, CARBOPLATIN, 5-FLUOROURACIL, PACLITAXEL AND DOCETAXEL) is included it still remains as a high economic cost and highly damaging treatment/alternatives[21, 22, 23].

CISPLATIN is a chemotherapy medication that belongs to the platinum-based antineoplastic family of medications and works in part by binding to DNA and inhibiting its replication. It is used to treat patients with bladder, ovarian, head and neck, lung, cervical, esophageal, and brain cancers[7]. Along with side effect like nephrotoxicity, neurotoxicity, ototoxicity, patients also exhibited resistance to the platinum based CISPLATIN[5]. Reduced accumulation of drug is a significant mechanism that results into resistance and reductions in accumulation of drugs by a factor of between 20% to 70% could cause resistance of CISPLATIN by a factor of 3 to 40 fold respectively[22]. Chemical anticancer drugs exhibited numerous unwanted side effects hence, anticancer drug obtained from plants that act against proliferating cancer cells was thought to be a suitable alternative.

Spices were in use since ancient times as a source of antimicrobial, anti-inflammatory, digestive and aromatic strategies and may be a key to determining the balance between pro- and anticancer factors that regulate risk and tumor behavior[10].

India, known as 'home of spices', produces about 75 spices and has emerged as one of the largest producer, consumer and exporter of spices in the world[23]. Various studies are currently in progress to search for natural-based antiproliferative and chemopreventive agents which can act as alternatives to the chemically-synthesised drugs and are potentially less toxic and contain lesser side effects. Of these agents spices are gaining importance.

Human Cancer cell lines are widely used to test emerging new therapeutic strategy in order to improve the efficacy of cancer treatment. Efficacy of anticancer properties of Anise seed extract is limited to human prostate cancer cell line PC-3 & gastric cancer cell line AGS. However, to the best of our knowledge anticancer property of anise seed extract on oral squamous cell carcinoma had not been explored.

Anise seeds are composed of various proteins, fatty and essential oils, crude fibres, starch, anethole, estragol, limonene, pinene etc. of which anethole, the main constituent of anise seed is said to possess anti-inflammatory and anti-tumor activities[24,25,26]. The procured KB cell lines were exposed to anise seed extract and chemotherapeutic drug CISPLATIN for 24 hours and cytotoxicity was determined by studying the morphological changes under an inverted phase contrast microscope and recorded as images and assessed using MTT assay.

Kadan et al. in 2013 found that anise seeds extract had an anticancer activity against human prostate cancer cell line PC-3 with notable effect even at low concentration[12]. Similarly, Rahamoz-Haghighi S and Asadi MS, in 2016 prepared and used an ethanolic extract of anise seed on gastric adenocarcinoma AGS cells by MTT assay. They found that extract inhibited the growth of cancer cells in doses between 15-480 µg/mL and its effect was in time dependent manner[13].

As OSCC is a common malignancy associated with recurrences it would be ideal to formulate a treatment strategy which ensures prognosis increases for the patient. There is a continuous quest of newer drugs promising effective results. One such emerging drug could be use of plant and plant based products which may be used as an adjunct along with current treatment modalities as they offer a good cytotoxic activity. Development of such novel techniques and newer strategies may ensure beneficial effects to patients thereby increasing their prognosis.

The current study showed promising cytotoxic properties of the anise seed extract thus prompting more in vivo researches for effective implementation.

Conclusion

In our study any rounding or shrinking of cells, granulation, vacuolization in the cytoplasm of the cells were taken to be the indicators of cytotoxicity.

On observation under the microscope we found a decrease in the percentage of viable cells with increase in concentration of anise seed and CISPLATIN extracts along with shrinkage and clumping of cells as the concentration gradient increased.

Using MTT Assay the viability of the cells expressed by purple formazan crystals were recorded where the intensity of the colour was measured as optical density OD values. The OD value were recorded for control as well as samples of increasing concentration of anise seed and CISPLATIN extract.

LC_{50} Value:

1. CISPLATIN- 30.0058µg/mL (Calculated using ED50 PLUS V1.0 Software)

2. Anise seed-48.0705 µg/mL

The reported results show that anise seed extract has significant anticancer effect on KB cell line. Thus, anise seeds could be a natural source of novel anticancer compounds with anti proliferative and/or apoptotic properties.

Due to its anticancer pharmacological effect, clinical trials are recommended to evaluate the beneficial effects of this plant in human models. It is necessary to further carry out more studies to assess the anticancer property of anise seed for its use as an adjunct to other chemotherapeutic drugs.

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