OIRIGINAL RESEARCH

Anti-cancer potency of garlic *(Allium sativum)* extract in comparison to 5-fluorouracil - An *in vitro* study.

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Abstract

Context: Garlic (*Allium sativum*) with its main component organosulfur compounds has an anticancer effect against a large variety of cancer cells. In this study we examined the anti-cancer activity of garlic extract on KB- cancer cell lines.

Aims: a) To determine the cytotoxic activity of garlic extract on KB cell line, b) To determine the cytotoxic activity of 5-FU on KB cell line, c) To compare the cytotoxic activity of garlic extract and 5 FU on KB cell line.

Methods and materials: An extract of ascending concentration was prepared using 5-FU and garlic. The KB cell lines were treated with both these extracts. The cytotoxic properties of the extracts were assessed by MTT assay by measuring the number of viable cells.

Results: The results showed that the percentage of viable cells is significantly correlated with different concentrations of garlic *(Allium sativum)* extract ($p<0.05$) and is significant with a p value of 0.0027.

Conclusion: Epidemiological observations and studies have indicated the anti-carcinogenic potential of garlic, traditionally used for various human diseases. This study aims to utilize this potential of garlic or its components as an adjuvant to an anti-cancer drug or in the development of new anticancer drugs.

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

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Introduction

Oral Squamous Cell Carcinoma (OSCC) is currently the 6th most common carcinoma occurring worldwide.[1] It is more common in Asian countries than western countries. It has a more male predilection. The Indian Council of Medical Research (ICMR) estimates that the country is likely to register over 17 lakh new cases and would report over 8 lakh deaths by 2020. Cancer requires a multi-targeted therapeutic approach. 5-Fluorouracil (5-FU) is one of the most commonly used drugs for treatment for carcinomas of breast, digestive tract, and other cancers including OSCC. The principle action of 5FU is that it inhibits the enzyme necessary for cell replication.[2] Myelotoxicity, cardiotoxicity and development of drug resistance are the major disadvantages of 5FU. 5FU target both the cancer cells and the healthy cells. This non-specific targeting along with the development of drug resistance are the main side effects of chemotherapy. Thus there is a need to overcome the disadvantages of the therapeutic strategies and to look into more safer anticancer drugs. But to date; such drug has not yet evolved. Alternatively, great effort has been devoted to find medicinal plant extracts that have an anticancer activity to be used as a co-adjuvant with anticancer drugs. Among these plants, garlic (*Allium sativum*) has long been known to have medicinal qualities and anticancer effect against pancreas, colon, stomach, liver and breast cancer.[3]

Weisberger and Pensky in 1958 first described the anticancer properties of garlic.[4] Garlic have a variety of functions like diaphoretic, expectorant, antispasmodic, antiseptic, bacteriostatic, antiviral, antihelminthic and hypotensive effects and hence it is commonly used to treat chronic bronchitis, recurrent upper respiratory tract infections and influenza.

In vitro studies on cancer cell lines is an essential pre requisite to any experimental drug before it can be used on clinical samples. Numerous cancer cell lines are available of which HeLa is the most commonly used cancer cell line. KB cell line is now known to be a subline of the ubiquitous KERATIN-forming tumour cell line HeLa.[5] It was originally thought to be derived from an epidermal carcinoma of the mouth, but was subsequently found, based on isoenzyme analysis, HeLa marker chromosomes, and DNA fingerprinting, to have been established via contamination by HeLa cells. The cells are positive for keratin by immunoperoxidase staining. KB cells have been reported to contain human papillomavirus18 (HPV-18) sequences.

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The MTT assay has been widely used to assess the cytotoxic property of the test samples by assessing the number of viable cells. The MTT assay is dependent on mitochondrial respiration and indirectly serves to assess the cellular energy capacity of a cell.[6] The MTT assay is a colorimetric test that can easily measure the number of viable cells. In our present study we examined the anti- cancer activity of garlic extract (ethyl acetate extract) and 5FU extract on KB- cancer cell lines by MTT assay method to assess the viability of cells.

Materials And Methods

Cell lines and cell culture.

We used KB cell lines in our current study as KB cancer cell line which was initially 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 cm2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO2 incubator (NBS Eppendorf, Germany).

Preparation of the extract

This current study we prepared ascending concentrations of aqueous extract of garlic (Extract 1) and 5FU (Extract 2).

Extract 1: The garlic was purchased, ground to a fine paste and mixed with ethyl acetate which was then kept in the open for partial evaporation in an extraction plate and extract was obtained and the necessary five concentration (6.25, 12.5, 25, and 50,100 µg /ml) solutions were prepared. The sample solutions were then filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

Extract 2: Similarly 5- fluorouracil was also serially diluted serially by two fold to obtain five concentration solutions.

Cell Treatment Procedure

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO2 incubator. After 24 hours the growth medium was removed, cells were treated with *garlic* extract of increasing concentrations (6.25, 12.5, 25, 50,100 µg/ml) in respective wells and incubated for 24 hours. Similar procedure was repeated with same concentration of 5FU extract on cultured KB cell.

Cytotoxicity Assay by Direct Microscopic observation:

The cells treated with ascending concentration extracts of garlic and 5FU were observed after 24 hours using 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.

Cytotoxicity Assay by MTT Assay Method:

15 mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS 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 37ºC 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 pipetting 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 percentage of growth inhibition was 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 calculated using ED50 PLUS V1.0 Software.

Results

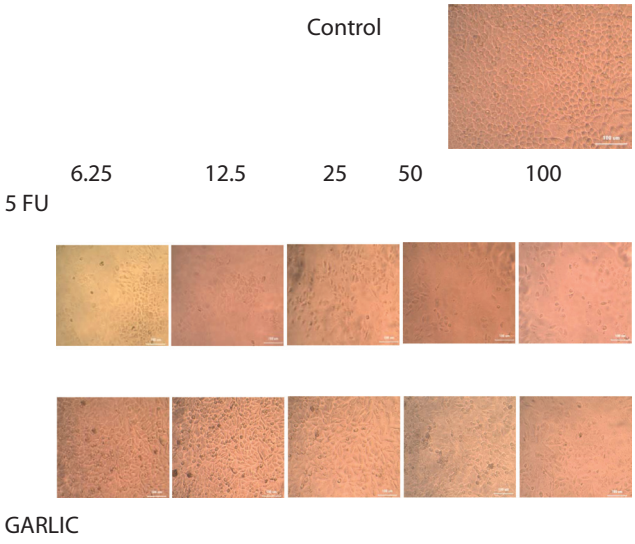
In the present study, the KB cell lines were incubated in ascending grades (6.25, 12.5, 25, and 50,100µg/ml) of garlic extracts and the cytotoxic activity of the extracts were assessed for cellular morphological changes under a microscope and measured using MTT assay. The LC_{50} value was calculated 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 garlic extract as well as 5FU 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.

The viability of the cells was expressed by formation of purple formazan crystals the intensity of which was measured as optical density OD values. The OD value was recorded for control as well

as samples of increasing concentration of garlic and 5FU extract.

Table 1 SAMPLE CODE: GARLIC EXTRACT

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	1.4208	1.3979	1.3764	1.3984	92.62
12.5	1.3108	1.3114	1.2247	1.2823	84.93
25	1.0152	1.0227	1.0302	1.0227	67.74
50	0.8670	0.8438	0.8426	0.8511	56.37
100	0.6990	0.6982	0.7085	0.7019	46.49

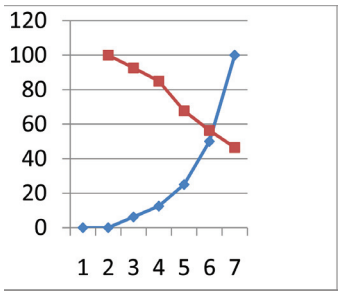
Table 2 SAMPLE CODE: 5-FLUOROURACIL

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	0.6620	0.6529	0.6774	0.6641	43.99
12.5	0.5025	0.5175	0.4846	0.5015	33.22
25	0.4072	0.4039	0.4196	0.4102	27.17
50	0.2895	0.2612	0.2534	0.2680	17.75
100	0.1907	0.1978	0.1984	0.1956	12.96

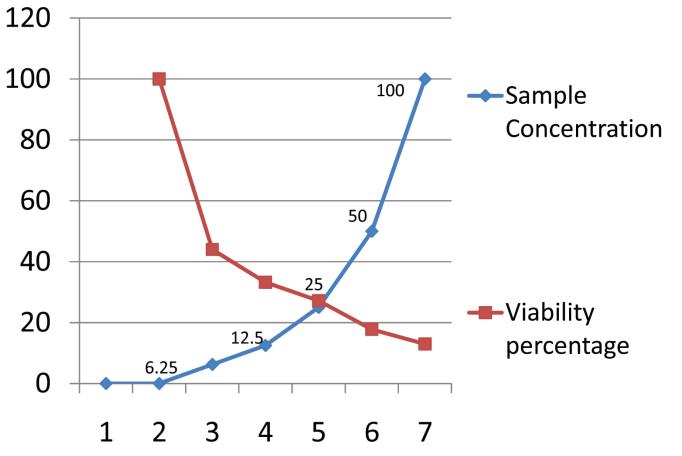
We noted that the viability of the cells decreased along with increase in the concentration of garlic extract i.e., the lowest concentration of garlic extract 6.25 the viability of cells were 92.62% whereas garlic extract concentration of 100 showed 46.49% viable cells.

Similarly we noted that as the concentration of 5FU extract increased the viability of the cells decreased i.e., lowest concentration 6.25 of 5FU extract showed 43.99% of viable cells and 12.96% of viable cells at 100 concentration.

In our study we observed that the average OD value decreased with increase in concentration of both garlic extract and 5FU extract which in turn represented a decrease in percentage of viable cells.



Garlic Extract



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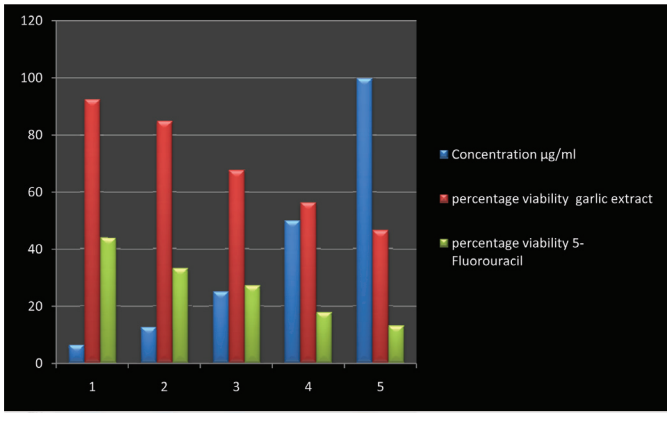
5-FU

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

Table 3 Comparison of viability percentage

Concentration µg/ml	Viability percentage of garlic extract	Viability percentage of 5-FU
6.25	92.62	43.99
12.5	84.93	33.22
25	67.74	27.17
50	56.37	17.75
100	46.49	12.96



Based on the percentage of viability, LC_{50} value was calculated.

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

In this current study,

LC_{50} for garlic extract is 81.177µg/ml

LC_{50} for 5FU extract is 5.57936 µg/ml

The values were then statistically analyzed by using Pearson Correlation Coefficient which showed a significant p value of 0.0027.

Discussion

Garlic contain many sulphur compounds (aliin, allicin, ajoene, allylpropyl disulfide, diallyl trisulphide, sallylcysteine, vinyldithiines, S-allylmercaptocystein, and others), enzymes (allinase, peroxidases, myrosinase, and others), amino acids (arginine and others), and minerals (selenium, germanium, tellurium and other trace minerals)[7]. Biological effects of garlic is due to its organosulfur compounds[8]. Allicin (diallyl thiosulphate), discovered by Cavallito and Bailey (1944) is responsible for garlic's typical pungent smell[9]. Allicin is not expressed in garlic unless it is crushed; injury to the garlic bulb activates the enzyme allinase, which metabolizes alliin to allicin[10,11]. Two major groups of compounds that show active anticancer effects have been known. One group is the lipid-soluble allyl sulphur compounds like diallyl disulfide (DADS) and diallyl trisulfide (DATS), and the other one is the water-soluble compounds like S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC) Thomson and Ali, 2003.

Several mechanisms have been proposed to explain the anticancer potency of garlic. These includes inhibition of mutagenesis, modulation of enzyme activities, inhibition of DNA adduct formation, free-radical scavenging, and effects on cell proliferation and tumor growth. Up to date several papers show that in cancer cell lines such as human colon adenocarcinoma (Ht29), human leukemia (U937), human colon cancer cell line (Colo 205), and mouse chronic myelocytic leukemia (32Dp210), garlic extracts caused ROS dependent cell death.[12,13,14,15] There are several studies reporting the antioxidant properties of different garlic extracts.[16,17] The contents of phenolic compounds are able to scavenge endogenous cellular ROS. However, in living cells those compounds can also exhibit prooxidant action and in that way increase expression of ROS metabolising enzymes and protect cells for a longer period of time against ROS. Those properties of compounds from garlic extracts may play an important role in cancer therapy by activating apoptotic processes where the basal level of ROS is already very high.[18,19]

In the present study, both garlic and 5-FU extract inhibited proliferation of KB cells in a reverse dose-dependent manner. The Cytotoxic effects of garlic extracts were statistically significant to the cytotoxic effects of 5-FU.

Similar results were obtained by Su et al. (2006) who showed that concentrations of 0.0005–0.002 mg/mL of garlic extracts caused caspase- 3 dependent apoptosis in Colo 205 cell line.[20]

In a study by MS Islam in 2011, 95% Hela cancer cell were destroyed at 500µl conc aqueous extract of garlic.[4]

Amira M Shaban (2018) et al founded anticancer activities for the four fractions with best effect for EtOAc fraction which had the lowest IC50 values 21.32 µg/ml in MCF7 and 26.22 µg/ml in HepG2.[3]

In contrast to the results of our study, Farrokh Farhadi et al (2015) stated that minimum conc(1µg/mL) of fresh garlic extract exerts a greater effect on the apoptotic activity in comparison to other higher doses.[20] In the study of Konrad A. Szychowski et al (2016), they found that no cytotoxic activity was there even with 1mg/ml of aqueous garlic extract SCC -15.[22] The results of our study conducted on garlic extract showed us it's potential cytotoxic effects, thus prompting more in vivo studies for it's effective implementation as an anticancer agent.

Conclusion

The results of the study revealed that garlic extract shows potent cytotoxic activity. The cytotoxic activity of garlic extract was noted to be statistically significant to the cytotoxic activity of 5-FU extract on KB cell line. Thus we imply that garlic extract or its main component could be used as an adjuvant to anticancer drug or can help in the development of new anticancer drugs. Garlic is potentially beneficial for cancer prevention, and as such is also recommended as a food supplement for cancer patients.

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