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The Efficacy of Phytochemical Allicin on Non-Enzymic Antioxidants and Cytotoxicity Induction in Cultured Breast Cancer Cell Lines

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Abstract
The study aims at analyzing the efficacy of garlic derived phytochemical Allicin, in elevating the non-enzymic antioxidant activity and its cytotoxic potential on its treatment to cultured breast cancer cell lines. In the experiments, cultured breast cancer cell lines (MCF-7 and T47D) were treated with allicin and through standardized protocols the cell lines were assayed for the activities of non-enzymic antioxidants by way of analysis of Hydroxyl radical scavenging activity and Superoxide radical scavenging activity as compared to untreated control. Furthermore, cytotoxic potential of allicin was also analyzed by MTT assay. It was found that allicin treatment significantly increased the activity of the said non- enzymic antioxidant activities as compared to control. It was also found that allicin has a significant role in inducing cytotoxicity in the said cell lines and shows a promising growth inhibition potential. This study demonstrates that Allicin treatment to the said cancer cell lines shows a significant antioxidant activity and a significant cytotoxic potential which proposes future application of the phytochemical in cancer therapy in way which should be cost effective, easily available, and free from side effects of the synthetic anticancer drugs. The study also proposes future research in developing targeted delivery of such herbal chemicals to treat the dreaded disease.

Keywords
Enzymic Antioxidants, Allicin, Cancer Cell lines, phytochemicals, Nutraceuticals

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INTRODUCTION
The ever-increased burden of stress arises from the challenging situations of day-to-day life, increasing pollution, poor nutrition etc., when remains unchecked, increases the risk for serious illnesses, including heart disease, high blood pressure, diabetes, cancer to name a few. Often it leads to other problems, such as infertility, ulcers, sleep disturbance, chronic fatigue, severe depression, overeating, memory loss, substance abuse and lowered productivity. Amongst these chronic expressions of stress, pollution and poor nutrition, cancer issues, is the most dreaded, now days.
Organisms are maintained by homeostasis, a delicate balance between cell proliferation and cell death. When this balance is disturbed, either by an

increased in proliferation or by decreased cell death, a tumor may be caused, which can further progress into a cancer. Tumor development may be described in terms natural selection followed by clonal expansion of normal cells resulting in monoclonal lumps originating from the progeny of a single cell (Nowell, 1976). Aberrations that provide growth advantages to the cell will accumulate during the clonal selection process. Despite clinical advances in early cancer detection and development of new anti-cancer drugs, healthcare and the high cost of cancer treatments causes obliteration of cancer diagnosis and effective treatment in low-income groups.

Allium species have been well known for its uses as spices and condiments along with its nutritional and medicinal values. Onion and garlic could be the first cultivated crops owing to their growing versatility, long storage time, and portability. They could be dried and preserved for times when food was scarce. The *Allium* family presently has over 600 members, distributed all over Europe, North America, Northern Africa and Asia. They differ in taste, form and color, but are close in biochemical, phytochemical and nutraceutical characteristics. The plants have multiple utilities namely as ornamentals, vegetables, spices, or as medicine. There are about 120 different documented uses of the *Allium* plants, and apart from their remarkable medicinal efficiencies, *Allium* plants are generally consumed for their flavors. Their nutritive and theraputic values have been appreciated by several workers. (Fenwick and Hanley, 1985).

Allium species have been chemically characterized and have been found to contain thiosulfinates and other organosulfur compounds. The thiosulfinates are the result of reaction catalysed by enzyme alliinase from their respective S-alk (en)yl cysteine sulfoxides. These chemicals are mainly responsible for flavor and odour of garlic and onion. These chemicals are also Lachrymatory factors that induce tears.

Garlic *(Allium sativum)* and its active chemical constituents have been widely used as preventive therapeutic agents against clinical conditions such as cancer, diabetes, cardiovascular diseases, and other metabolic diseases such as atherosclerosis, hyperlipidemia, thrombosis, hypertension, and dementia [Lawson LD 1998]. Experimental and clinical studies by researchers worldwide indicate that garlic reduces the cancer risk by bringing about inhibition of carcinogen activation and/or enhancing the activity of detoxification systems to neutralize of activated carcinogens and showing antitumor potentials [Milner JA. 2001].

With the increasing interest on the utilization of natural bioactive compounds for various purposes these garlic derived nutraceuticals became subjects of active research regarding their potency as food preservatives and therapeutically active chemicals. This piece of research work examines the biological activities of *Allium* thiosulfinate Allicin as a potent anticancer nutraceutical.

METHODOLOGY

Maintenance of Cell Lines

The MCF-7 and T47D breast cancer cell lines were purchased from Sigma Aldrich and were maintained in DMEM (Dulbecco's Modified Eagle's medium) Sigma Aldrich kit according to the manufacturer's instructions. All the cell lines were maintained in the media supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine in a humidified atmosphere of 5% CO_2 and 95% air at 37° C. The assays and estimations were done on the said cultured cell lines by treatment with various concentrations of Allicin as compared to untreated control.

1. ANALYSIS OF NON ENZYMIC ANTIOXIDANT:

The standard methodologies for analyses of non-enzymic antioxidants in untreated controls and allicin treated cultured cell lines were taken up and are presented under the following subheadings marked A and B:

A. Hydroxyl radical scavenging activity (Elizabeth and Rao, 1990).

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell.

B. Super oxide anion radical scavenging activity (Winterbourn et al. (1975)

Superoxide dismutase (SOD) is a metalloenzyme that catalyze the dismutation of superoxide radical into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) and consequently provide an important defense mechanism against superoxide radical toxicity.

2. Analysis of Cytotoxicity by MTT Cell Proliferation Assay (Igarashi and Miyazawa, 2001)

The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.

Statistical Analyses

Numerical data are represented as Mean=SEM of five independent experiments (N=5). Statistical significance of the difference from untreated (control) values at $p<0.01$ determined by unpaired T-test of two sample unequal variance using GraphPad software.

RESULTS OF ANALYSIS OF NON ENZYMIC ANTIOXIDANT:

The results of non-Enzymic antioxidant analysis are presented under the following subheadings marked A and B:

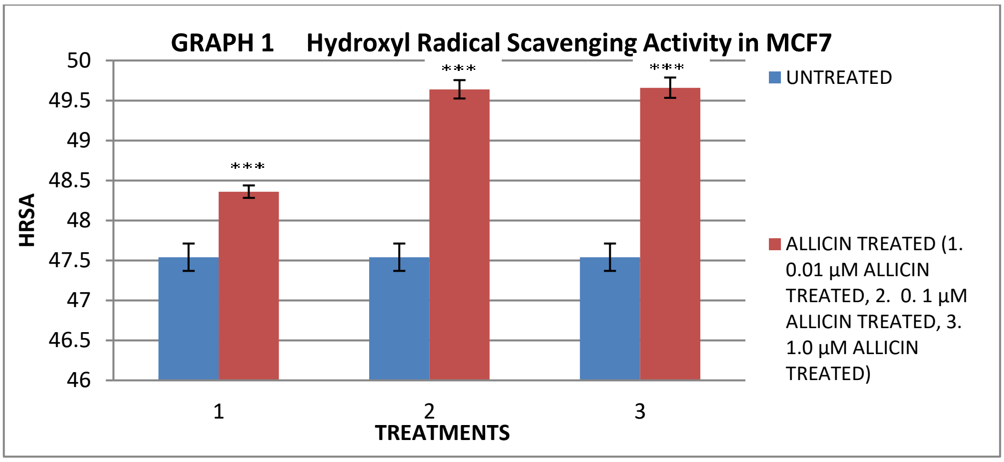
A. ANALYSIS OF HRSA (hydroxyl radical scavenging activity)

The results of the analyses of HRSA in the MCF-7 and T47D cell lines are presented as Table 2 followed by Graph 3 (for MCF-7) and Graph 4 (for T47D).

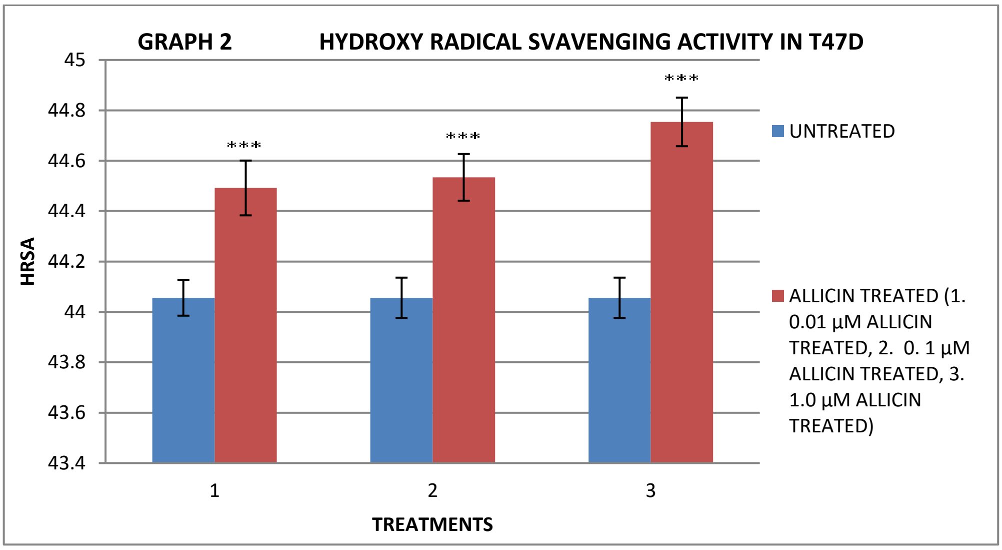
TABLE 1- Hydroxyl Radical Scavenging Activity in Untreated and Allicin treated cell lines (MCF-7 and T47D)

	UNTREATED (CONTROL)	0.01 µM ALLICIN TREATED	0.1 µM ALLICIN TREATED	1.0µM ALLICIN TREATED
HRSA MCF7	47.54±0.171077	48.36±0.07791***	49.64±0.114924***	49.66±0.12721***
HRSA T47D	44.056±0.109977	44.492±0.108761***	44.534± 0.09253***	44.754±0.096509***

GRAPH 1 AND 2: Hydroxyl Radical Scavenging Activity in Untreated and Allicin treated cell lines (MCF-7 and T47D)



Numerical data are represented as Mean=SEM of five independent experiments (N=5) (p<0.01)



Numerical data are represented as Mean=SEM of five independent experiments (N=5) (p<0.01)

The results (Table5 and Graphs 9 & 10) of the analyses of Hydroxyl Radical Scavenging Activity_in untreated and Allicin treated cell lines show statistically significant increase with increasing

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concentrations of Allicin (i.e 0.01 µM, 0,1 µM and 1.0 µM). Numerical data are represented as Mean=SEM of five independent experiments (N=5).

B. ANALYSIS OF SRSA (Superoxide radical Scavenging activity)

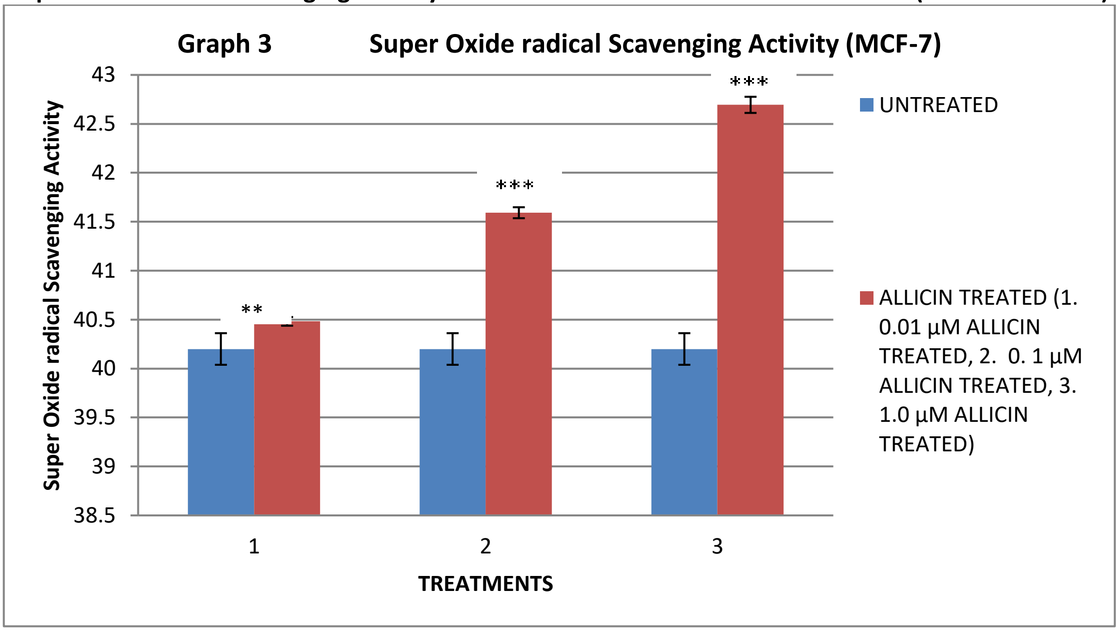
The results of the analyses of SRSA in the MCF-7 and T47D cell lines are presented as Table 2 followed by Graph 3 (for MCF-7) and Graph 4 (for T47D).

TABLE 2- Superoxide radical Scavenging activity in Untreated and Allicin treated cell lines (MCF7 and T47D)

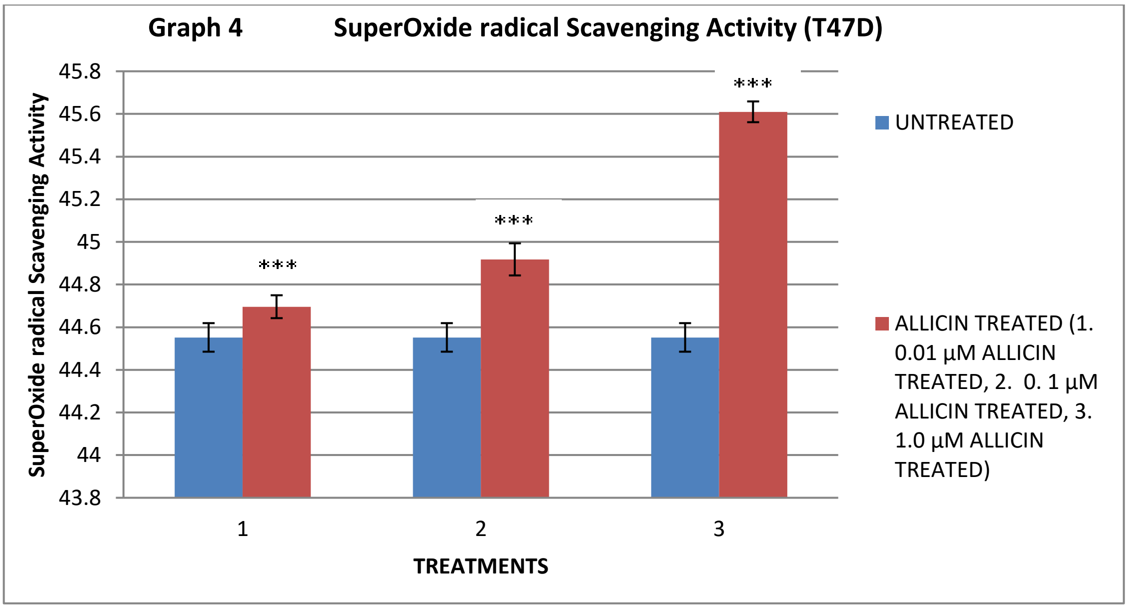
	UNTREATED(CONTROL)	0.01 µM ALLICIN TREATED	0.1 µM ALLICIN TREATED	1.0µM ALLICIN TREATED
SRSA IN MCF-7	40.2±0.1614	40.482±0.044215**	41.592±0.055946***	42.694±0.08228***
SRSA IN T47D	44.552±0.067026	44.696±0.053689***	44.918±0.075283***	45.61±0.048734***

GRAPHS 3 AND 4

Superoxide radical Scavenging activity in Untreated and Allicin treated cell lines (MCF7 and T47D)



Numerical data are represented as Mean=SEM of five independent experiments (N=5) (p<0.01)



Numerical data are represented as Mean=SEM of five independent experiments (N=5) (p<0.01)

The results (Table 6 and Graphs 11 & 12) of the analyses of Superoxide Radical Scavenging Activity_in untreated and Allicin treated cell lines show statistically significant increase with increasing concentrations of Allicin (i.e 0.01 µM, 0,1 µM and 1.0 µM). Numerical data are represented as Mean=SEM of five independent experiments (N=5).

RESULTS OF ANALYSIS OF CYTOTOXICITY

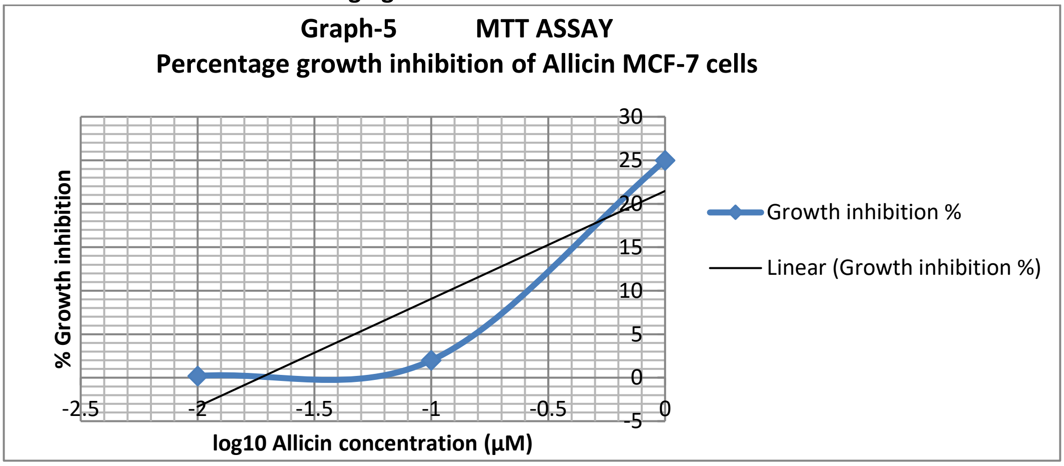
In the present study MCF-7 and T47D cells showed growth inhibition in a dose dependent manner when treated with Allicin at concentrations 0.01, 0.1 and 1.0 µM. The percentage of dead cells for each concentration and IC50 values are indicated in the Tables10-13 and graphs 14-17.

TABLE 3– MTT ASSAY (MCF-7)

S.No.	Allicin Concentration (µM)	log $_{10}$ Allicin Concentration (1µM)	Growth inhibition %
1	0.01	-2	0.2
2	0.1	-1	2
3	1	0	25

GRAPH 5

Percentage growth inhibition of Allicin MCF-7 cells

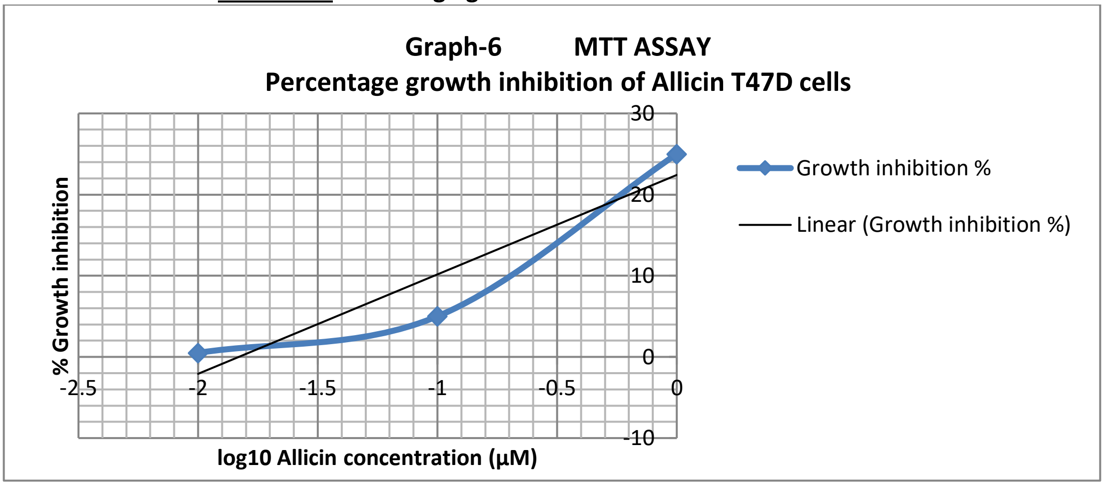


IC50 from the graph for MCF-7 = 7.94

TABLE 4 – MTT ASSAY (T47D)

S.No.	Allicin Concentration (µM)	log $_{10}$ Allicin Concentration (1µM)	Growth inhibition %
1	0.01	-2	0.5
2	0.1	-1	5
3	1	0	25

GRAPH 6: Percentage growth inhibition of Allicin T47D cells



IC50 from the graph for T47D = 6.91

DISCUSSIONS

As already described Allicin (diallyl thiosulfinate or diallyl sulphide) as a biologically active compounds found in crushed garlic due to stress induced activation of an enzyme called allicinase that converts its precursor, Alliin (s-allyl cysteine sulphoxide) into Allicin (Block 2010). Studies throughout the world on the efficiency of allicin during the last two decades have shown its overwhelming potential to act as a cure for many chronic degenerative diseases that had threatened mankind since long. The work emphasizes the utility of this multifaceted Nutraceutical Allicin on cancer.

The terms “free radicals” and “active oxygen” have gained the interest of clinicians and researchers throughout the globe.

Human beings have not only created conditions as a way of their scientific and industrial development. that cause oxidative stress but also have created a lifestyle by consuming and using substances like alcohol tobacco etc which have multiplied their levels of oxidative stress manifold. None the less oxidative stress also arises from intake of drugs (including anticancer drugs) that are common in today’s medical practice. Apart from the exogenous sources of oxidative stress, endogenous sources include the products of metabolic activities of mitochondria or microsomes, peroxisomes etc. Reactive oxygen radicals may be involved in cancer development. The type of active oxygen species involved, and the intensity of stress decides mechanism and degree of oxidative stress (Mates JM et al 1999).

It is well reported that aged garlic extract containing lipid soluble organosulphur organic chemical components that include S-allylcysteine and S-allylmercaptocysteine have antioxidant action by increasing activity of cellular antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and GPx (Borek 2001). Garlic has been long thought to elicit anticarcinogenic effect through a number of mechanisms i.e free radical scavenging activation of antioxidant defense enzymes (glutathione S-transferase, catalase) and activating DNA repair mechanisms etc (Liu J et al., 1992). Moreover it has been shown that over expression of catalase in the prostrate cancer cell lines DU145 protects against apoptosis induced by DATS (Xiao et al., 2004).

The results of the studies by A. Tiwari 2001, proposes that free radicals are fundamental to any biochemical process and form an essential constituent of metabolic pathways under aerobic conditions. The reactive oxygen species play an important role in the genesis of several oxidative stress related diseases like carcinogenesis, cardiovascular disease, rheumatoid arthritis, ulcerative colitis, and neurological degenerative diseases (B. Halliwell, J.M. Gutteridge 1990). Among the free radicals studied within physiological system hydroxyl radical is the most reactive oxygen centered species and causes severe damage to adjacent biomolecules. In connection to the above, novel natural plant derived antioxidants have been extensively studied throughout the globe for their antioxidant and radical scavenging properties (B. Sangameswaran et al., 2008). Studies by Ames BN. 1983, that damage to DNA by ROS has been widely implicated as a major player in the genesis of cancer making ROS potential carcinogens because of their ability to cause promotion and progression. The growth promoting effects of ROS can be said to be due responsive cell signaling cascade that the affect. Several mutations are caused by oxidative damage of DNA that include a range of specifically oxidized purines and pyrimidines, alkali labile sites, single strand breaks and instability formed directly or during repair process for such damages (Breen AP, Murphy JA, 1995). Since ROS brings about a large assay of DNA modification it has been difficult to establish the frequency and specificity of mutations by individual oxygen radical induced lesions. Moreover, some of these modified bases have been found to possess mutagenic properties imparting the abilities that causes carcinogenesis. Several studies show that ROS can cause damage to all bases, but mutations are usually related to modification of GC base pairs, while that of AT base pairs rarely leads to mutations (Wang D et al., 1998). Such mutations are usually base pairs substitutions, whereas base deletions and insertions are less prevalent. Studies have shown that in human tumors, G to T transversions from the most frequent mutations in the p53 suppressor gene (Hollstein M *et al.*, 1991). Moreover, studies related to the use of single stranded DNA template in a sensitive foreword mutations system different mutation such as the tandem double CC → TT substitution have been observed in DNA under the influence of oxygen radicals (Reid TM, Loeb LA, 1993). Studies throughout the globe show that increase of modified bases in cancerous tissue may be due to the production of large amount H_2O_2 a pathological characteristic of the process of transformation in humans (Olinski R 1998). In connection to these initiation of carcinogenesis in humans by reactive oxygen species is further supported by the presence of oxidative DNA modifications in cancer cells (Poulsen HE et al., 1998).

In accordance with the mentioned studies and others the present study demonstrates that Hydroxyl

Radical Scavenging Activity in untreated and allicin treated cell lines show statistically significant increase with increasing concentrations of allicin (i.e. 0.01µM, 0.1 µM, 1.0 µM) which intends to suggest that antioxidative properties of the plants are becoming a boon due to their possible uses as natural additives to replace synthetic ones.

Various pathways generate various reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion radicals (O_2), and hydroxyl radicals (OH). (Kamata, H., and Hirata, H. 1999) suggested that many cellular enzymatic complexes have been found to be involved in the ROS production, such as NADPH cytochrome P450 reductases, hypoxanthine/xanthine oxidase, NADPH oxidase, lipoxygenase, per-oxidases, and cyclooxygenases. In connection to this mitochondrion are believed to be a major source of ROS in aerobic cells (2, 3). During the process of oxidative phosphorylation1–2% electrons that are transported escape and reduce O_2 to O_2 (Pani, G., et al 2009). Chronic oxidative stress has been found to be frequently associated imbalanced intracellular redox status, leading to a variety of pathological, such as malignancies, type II diabetes, atherosclerosis, chronic inflammation, cardiovascular diseases, and many neurodegenerative diseases (Droge, W. 2002).

With reference to the above several dietary polyphenolic compounds have been well investigated with respect to antioxidant potential and mechanism of action have been described (Khan, N., Afaq, F., and Mukhtar, H. 2008). A rise in free radicals through an increased in production of a defect in their removal causes damage in DNA, protein or lipid metabolism that can play a critical role in development of cancers. Moreover, small changes in redox conditions in the physiological systems can play a role in tumor progression by affecting cell growth or survival.

Studies pertaining to analysis have suggested that chronic oxidation induced damage is associated with cancer development and progression. In connection to the above it has been shown that ulcerative colitis is linked with higher incidence of colorectal cancer or chronic gastritis caused by infection of *Helicobacter pilori* (Nock, N.L et. al, 2006). Different levels of reactive oxygen species can activate different signaling pathways such that low or transient levels of ROS can activate signaling pathways towards proliferation of cells (Sundaresan, M *et. al*, 1995). Therefore, the redox control of cell growth involves antioxidants affecting the cellular growth in physiological conditions. Two types of antioxidants have been found to play a role in cancer such as exogenous antioxidants consumed through diet as well as the endogenous antioxidant enzymic and non-enzymic which contribute to control normal cellular redox status. Furthermore, animal, and cellular models have been used throughout the globe to analyses new class of chemo preventive and therapeutic agents such as flavonoids, polyphenols or isothiocyanates [Surh, Y.J. 2003]. These are the active components of dietary phytochemicals that play an important protective role against cancer such as dietary substances are curcumin, genistein, resveratrol, diallyl sulfide, S-allyl cysteine, allicin, lycopene, capsaicin, diosgenin, 6-gingerol, ellagic acid, ursolic acid, silymarin, anethol, catechins, eugenol, isoeugenol, dithiolones, isothiocyanates, indole-3-carbinol, isoflavones, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, Vitamin C, d-limonenen, lutein, folic acid, beta carotene, selenium, vitamin E, flavonoids and dietary fiber [Aggarwal, B.B.; Shishodia, S 2006].

In connection to the mentioned studies our work shows statistically significant increase of Superoxide Radical Scavenging Activity in untreated and Allicin treated cell with increasing concentrations of Allicin (i.e 0.01 µM, 0,1 µM and 1.0 µM). Hence Allicin, the organosulphur compound of garlic can be a future prospect to cobble for the cure of breast cancer.

The basis MTT assay is the reduction of tetrazolium salt by mitochondrial reductase such as succinate drawbacks for the evaluation of the toxicity of a given test substance. The MTT assay presumes that the respiratory activity is related to the cell viability. It has been worked out that allicin is able to inhibit many Cysteine containing enzymes (Wills E.D. 1956) and getting mitochondrial reductase with Allicin causes inhibition that might lead to less MTT reduction inspite of the fact that the cells are still viable. Alternative viability assays based on membrane permeability (e.g., propidium iodide) are also not suited as allicin is membrane active and forming transient pores in membranes(Gruhlke M.C.H et al., 2015).Nevertheless studies have shown that allicin treatment lowered MTT reduction in all cell lines under examination in a dose-dependent manner (Bernas T., Dobrucki J., 2002).Throughout the globe Epidemiological studies indicates that dietary factors influence the development of breast cancer and results of various experiments suggest that natural or synthetic constituents of diet have an ability to act as that inhibit breast cancer. In connection to this it has been found that among dietary factors, certain phytochemicals present in the daily diet have efficiency to elicit chemo preventive properties (Surh, Y.J. 1998). It was shown that allicin imparts an inhibitory effect on the ultrastructure of human hepatoma cells, (Zhong N *et* *al*., 2002) . They found that treatment of Vitro cultured hepatoma cells with allicin in this dose dependent manner intercellular Junction reduced in majority of cells causing cytoplasm shrinkage and marginal irregularities and increasing number of followed by appearance of apoptotic bodies. Experimental doses of allicin were found to cause disappearance of cell surface microvilli and decrease of cell motility related to organelles and organization of intra cellular microfilaments and microtubules. This suggests that Allicin may also have a specific inhibitory action against ability of hepatoma cells to metastasize. In connection to the above experiments on in vitro culture showed oil soluble compounds such as DADS, diallyl sulphide and diallyl trisulfide were efficient in causing inhibition of proliferation in CMT-13 canine mammary tumor cells while the water soluble compounds S-allyl cysteine, S-ethyl cysteine and S-propyl cysteine had little or no inhibitory effect (Sundaram, S.G and Milner, J.A. 1995) : S-allylmercapto cysteine present in a low concentration in aged garlic extract was effective in lowering the growth activities of MCF-7 human breast cancer (Sigounas, G. et al.,1997). Essential oils of garlic have been shown to contain ~60% DADS (9). Moreover, studies show that have the highest efficiency in reducing the growth of human tumors cells that are derivatives of (Sundaram, S.G and Milner, J.A. 1996). DADS is an oil soluble organosulphur compound found in processed garlic that has been shown to effectively inhibit the growth of human breast cancer cells in artificial culture. DADS has been found to inhibit canine (CMT-13) (Sundaram, S.G and Milner, J.A. 1993) and human (MCF-7 and T47D) (Lea 1999) breast cancer cell growth in vitro.

With reference to the above studies our study on MCF-7 and T47D cells showed growth inhibition in a dose dependent manner when treated with Allicin at concentrations 0.01, 0.1 and 1.0 µM. Our study shows that the percentage inhibition of the above mentioned cell lines was highest at the 1.0 µM dose of allicin and also shows an increase in the percentage inhibition on increasing dose. The results of this study reveal MCF-7 and T47D cells were sensitive to allicin. Despite many reports on inhibitory effects of garlic on cancer cell line, our data showed these effects are dose dependent. Further studies on animal models and humans are needed to clarify the important molecules and their mechanisms.

The ability to down regulate tumor cell proliferation by organosulfur compounds of Allium species has been reported in several studies using different cell cultures, including canine mammary tumor cells (Sundaram and Milner, 1993), human colon, lung, and skin tumor cell lines (Sundaram and Milner, 1996), human neuroblastoma cells (Welch et al., 1992), human and murine melanoma cells (Takeyama et al., 1993), and human prostatic carcinoma cells (Pinto et al., 1997).

Our findings intend to encourage the development of chemical derivatives of garlic and other allium species that are more potent in terms of their chemo preventive and therapeutic potential and suggest that allicin and related compounds might provide a novel cost effective and safe approach against cancer progression. Although this requires more advance work using the in vivo animal model systems. However, more of comparative and analytical in vitro and in vivo investigations are required to suffice the understanding of precise mechanisms regulating the antiproliferative effects and apoptosis induced by garlic and its chemical constituents such as allicin.

CONCLUSION

The results of our study strongly indicate that Allicin treatment to cultured cancer cell lines increase the antioxidant activity and effectively inhibit proliferation of the said cancer cells. Although further studies are required regarding mode of delivery of allicin to cancerous growths, but the study encourages the potential use of garlic derived allicin as a phytochemical for chemotherapeutic purposes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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