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ORIGINAL ARTICLE

Alliin the Precursor of Allicin in Garlic Extract Mitigates Proliferation of Gastric Adenocarcinoma Cells by Modulating Apoptosis

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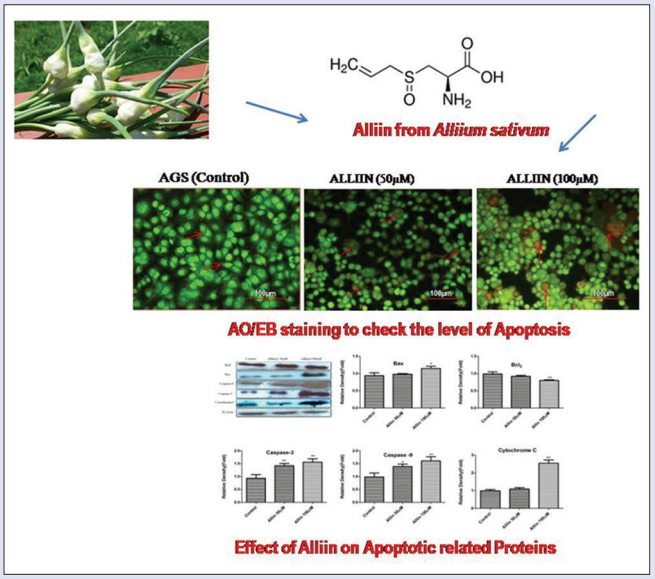
ABSTRACT

Background: Garlic, a common spice used since time immemorial for various purposes, is considered a potential functional food as it exhibits cardioprotection to chemoprevention properties due to the presence of organosulfur constituents in each garlic pod. Alliin is not widely studied for its bioactivity as it is an unstable compound which is converted to allicin on mechanical and chemical degradation. **Objective:** Hence, in the present study, the influence of alliin on gastric adenocarcinoma (AGS) cells and normal intestinal cells (INT-407) would be tested for its potent antiproliferative effect and mechanism of action. **Materials and Methods:** The quantity of alliin in fresh and dried garlic extract was measured by high-performance liquid chromatography to corroborate the cytotoxicity activity identified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and acridine orange/ethidium bromide staining. Further, to identify the mechanism of action, DNA fragmentation assay, annexin V Assay, and flow cytometry analysis of apoptosis followed by expression of apoptotic proteins such as Bax, Bcl-2, and cytochrome-C by Western blot was done. **Results:** It was identified that alliin inhibited proliferation of gastric carcinoma cells by decreasing the cell viability but not in the normal intestinal cells. The level of apoptosis was modulated by reactive oxygen species generation and decrease in mitochondrial membrane potential mediated by deregulation of Bax/Bcl-2 level at protein level leading to upregulation of Cytochrome C. **Conclusion:** It is impressive to note that alliin content was high in fresh aqueous extract compared to that of dried garlic extract which concludes that the use of garlic from time immemorial is a worthy functional food in its fresh form to combat cancer cells. However, *in-vivo* studies are warranted.

Key words: Alliin, apoptosis, DNA fragmentation, gastric adenocarcinoma, gastric cancer, reactive oxygen species

SUMMARY

- Alliin a precursor of allicin in garlic extract exhibits anti-proliferative potential in gastric adenocarcinoma cells
- The bioactivity is mediated by apoptosis which was modulated by reactive oxygen species generation, alteration of membrane potential through extrinsic and intrinsic pathway
- However, this was not noticed in normal intestinal cells proving it to be a phytonutrient of functional properties used from ancient times.



Abbreviations used: AGS-Gastric adenocarcinoma; ROS-Reactive oxygen species; MMP-Mitochondrial membrane potential; TBS-Tris buffered saline.

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INTRODUCTION

Cancers account for around 8.2 million deaths worldwide making it the major reason for death.[1] Although the symptoms and signs of stomach cancer are observed only in the advanced stage, stomach cancer ranks the second-most prevalent cancer among males and third-most among females both globally and in Asia.[2] More than 70% of stomach cancer cases occur in developing countries which accounts for a 5-year survival rate of <30% with half the world's total cases occurring in Eastern Asia as compared to 20% in developing countries.[2-4] Among the different types of stomach cancers, gastric cancers are more prevalent since a wide range of factors such as diet, lifestyle, age, and genetic factors influence the susceptibility to gastric cancers. Consumption of natural foods which includes fruits, vegetables, spices, nuts, and beverages such as wine and

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tea has influenced the prevention and management of cancers due to the presence of specific phytochemicals which attenuates pathologies involved in the disease process. These foods have been referred to as functional foods as they exhibit some potential health benefits and have been popular in recent years.

From ancient times, herbs and spices have been traditionally used for their medicinal properties and their flavor enhancement characteristics. Hence, of late, these spices have gained an impelling interest among researchers for multiple health benefits, due to the rising prevalence of chronic diseases.[5,6] Garlic (*Allium sativum*) has long been used as a condiment for flavor and as a therapeutic agent for its potential health benefits for both preventing and curing disorders in many nations around the globe.[6] Garlic possesses a number of phytoconstituents such as the sulfur-containing compounds alliin, ajoene, diallyl polysulfides, vinyldithiins, *S*-allyl cysteine, and unique non-sulfur compounds such as allixin and saponins.[7-9] Allicin is the principal bioactive compound derived from alliin in garlic produced as a result of activation of the enzyme alliinase after crushing or chopping of raw garlic. It primarily contains sulfur as the main constituent which on breakdown gives the characteristic odor and has been proven for its multifaceted therapeutic applications.[5]

Although there are many scientific reports on the role of allicin for its potential bioactivity from cardioprotection[10] to chemoprevention,[11,12] the present study aims to identify the possible antiproliferative activity of alliin the precursor of allicin and its level in fresh and dried garlic. Furthermore, the mechanism of its cytotoxic potential is deciphered on gastric adenocarcinoma (AGS) cells confirming it to be a molecule responsible to consider garlic as a functional food of therapeutic importance.

MATERIALS AND METHODS

Chemicals and other reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), DMSO, propidium iodide (PI) and trypsin, Kaighns F-12 modification media, penicillin, streptomycin, fetal bovine serum, and other cell culture supplies were purchased from Himedia Laboratories, Chennai. Acridine orange/ethidium bromide (AO/EB) was also obtained from Himedia Laboratories. Alliin and Annexin V Apoptosis Detection Kit were purchased from Sigma (St. Louis, MO, USA).

Quantification of alliin in garlic

About 500 g of fresh garlic (*A. sativum*) purchased from a local supermarket (variety originally from Pondicherry was peeled off completely and cut into small pieces for extraction. Simultaneously, 500 g of peeled and chopped garlic was shade dried, ground into a coarse powder and extracted with water (100 mg/ml), agitating overnight at room temperature. Then, the aqueous extracts were filtered and concentrated in a rotary evaporator at <40°C under vacuum, and the percentage yield of whole aqueous extract of fresh garlic and dried garlic was calculated. Before quantification, undissolved material was removed by centrifugation at 5000 g for 10 min. The supernatant was then filtered through a 0.45 µm filter and used for analysis.

Alliin, the abundant phytoconstituent both from fresh and dried garlic, was quantified by HPLC (Shimadzu LC solution). The aqueous extracts were made to known volumes and an aliquot was then injected into C18 reverse phase HPLC column for the quantification of alliin. A solution of 0.1% trifluoroacetic acid (TFA) in H_2O as mobile phase A and a solution of 0.1% TFA in acetonitrile was used as mobile phase B at a flow rate of 0.8 ml/min. The UV detection was performed at 210 nm.[13,14] The peak area of alliin in the extracts matched with standard alliin purchased from Sigma identified the quantity of alliin in both the fresh and dried garlic extracts.

Cytotoxic potential of bioactive lead from garlic

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Human AGS cell line and normal intestinal cells (INT-407) used for this study were purchased from the National Centre for Cell Science, Pune and cultured at 37°C in a humidified atmosphere with 5% CO_2, in Ham-F 12, Kaighn's modification medium supplemented with 10% fetal bovine serum (100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate).

MTT assay was carried out to check the cell proliferation which measures a purple formazan compound produced by viable cells.[15] Flat-bottomed, 96-well microtiter plates (4×10^3 cells/6.4-mm-diameter well) was used to carry out this assay. After 24 h, cells were treated with DMSO (0.1%–0.3%) or increasing doses of alliin. Then, the cells were treated with 50 µl of MTT reagent for 2 h at 37°C after 24 h of treatment and then treated with 100 µl of solubilization solution (DMSO) at 37°C. Formazan product formed was quantified using a microplate reader at 570 nm. Results were expressed as percentage of growth, with 100% representing control cells.

Analysis of cell morphology by acridine orange/ethidium bromide staining

The effect of alliin on cell death was also determined by AO/EB dual staining.[16] The cells were seeded in a six-well plate at a density of 1×10^3 cells per well and incubated overnight, followed by addition of various concentrations of alliin. After incubation, the media was removed and cells were washed with ice cold PBS. The cells were resuspended in 5 µL of AO (1 mg/mL) and 5 µL of EB (1 mg/mL). The morphological changes of the stained cells which reveal apoptotic cells were then observed using a fluorescence microscope and digitalized using an image analyzer.

DNA fragmentation analysis

To study the DNA fragmentation in AGS cells, control and alliin-treated cells were resuspended in lysis buffer (0.5% Triton X-100, 25 mM EDTA, and 10 mMTris-HCL, pH 8.0, 0.5% SDS) and incubated at 65°C for 1 h followed by addition of Proteinase K. DNA was then extracted with phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) and then centrifuged at 12,000 g for 15 min. DNA was then finally extracted with phenol and precipitated with ethanol, and subsequently allowed to air dry. Required amount of RNAase was added and incubated for 1 h. Finally, DNA was resuspended in Tris/EDTA buffer (10 mM Tris-HCL, pH 8.0, and 1 mM EDTA). Usually, 10^6 cells yielded 4–5 µg DNA. About 30 µl of the extracted DNA was then mixed with 3 µl loading buffer (TAE). The DNA fragmentation was visualized using 1.5% agarose gel, stained with ethidium bromide, and visualized by UV light and photographed by PhotoDoc-Imaging system.

Apoptotic potential of alliin

Annexin V assay

To further confirm apoptosis, annexin V-PE-based immunofluorescence was used to determine the percentage of cells actively undergoing apoptosis.[17] Briefly, AGS cells were treated with either DMSO (0.1%–0.3%) or alliin (100–200 µM). After 24 h of treatment, both adherent and floating cells were harvested and resuspended with 1X Annexin-V binding buffer and then double-labeled with annexin V conjugated with fluorescein isothiocyanate (FITC) and PI. Stained cells were analyzed using a Guava

easyCyte 8HT flow cytometer (Millipore, USA). Distribution of cells in early and late phases of apoptosis was evaluated using Incyte software is Guava® InCyte™ software by Merck(Millipore, USA).

Role of alliin on cell cycle distribution

PI staining was used to analyze DNA content. Cells were plated in a six-well plate for 24 h with different doses of alliin treatment. Cells were washed twice with PBS, fixed in 1 ml of 70% ethanol and stored at −20°C. Cells were then labeled with PI (0.05 mg/ml) in 1 ml of PBS containing 0.1% of Triton-X-100 and 50 μg of ribonuclease for 30 min in dark at room temperature and analyzed for DNA content using a Guava easyCyte 8HT flow cytometer (Millipore, USA) excited with blue laser (488 nm). Cells in different phases of cell cycle were evaluated using Incyte software.

Influence of alliin on ractive oxygen species and Mitochondrial membrane potential

Intracellular reactive oxygen species (ROS) was assessed with DCFDA (2,7-dichlorofluorescein diacetate) staining[15] to identify the mode of action of alliin. Cells were seeded at a density of 2×10^5 cells ml^{-1} in 6-well plates with different concentrations of alliin treatment. After 24 h of treatment, cells were washed with PBS and resuspended in 1 ml of complete media containing 10 μM DCFDA. After incubation of 30 min in dark, fluorescence was measured by flow cytometer at an emission of 488 nm to measure the level of ROS produced.

To determine the changes in the mitochondrial membrane potential ($\Delta\Psi m$), approximately 2×10^5 cells/well of AGS cells in a 12-well plate were treated with 50–200 μM alliin and incubated for indicated time periods. Cells were harvested by centrifugation, washed twice in PBS, and then re-suspended in Rhodamine-123 (4 μM). The cells were then incubated at 37°C in dark for 30 min and immediately analyzed by flow cytometry as previously described. The green signals were measured at E × 485/Em 535 nm.

Western blot analysis

Immunoblot of apoptosis-related proteins was done by a standard protocol.[18] After treatment of AGS cells with respective concentrations of alliin, cells were lysed with RIPA buffer and centrifuged at 12000 g for 30 min. The supernatant collected was checked for total protein concentration. Then, equal amount of protein (50 μg) was resolved on a 12% SDS-polyacrylamide gel for electrophoresis and then transferred to PVDF membrane by a semidry transfer unit (Hoefer). Membranes were blocked with Tris-buffered saline (TBS) containing 5% nonfat dry milk for 2 h to avoid nonspecific binding sites and incubated with 1:1000 dilution of primary antibodies (β-Actin, caspase-9, caspase-3, Bax, Bcl-2, and cytochrome-C from Santa Cruz Biotechnology, USA) overnight, and then washed with TBS containing 0.1% Tween-20 and incubated with HRP-conjugated secondary antibody at 1:2500 dilution for 90 min at room temperature. After proper washing with TBS containing 0.1% Tween-20 for three times, the transferred proteins were visualized on X-ray photographic film and quantified by Imaje J software is NIH Imaje J 1.5Oi.

Statistical analysis

Data were analyzed using ANOVA test with the $P < 0.05$ (*) and $P < 0.01$ (**) levels of significance and interpolation of results were performed using GraphPad Prism software, and the results were expressed as mean ± standard error of the mean $P < 0.05$ was considered significant.

RESULTS

Chemical analysis of garlic extracts

In the present study, the content of alliin, a sulfoxide present in both fresh and dried garlic was estimated. Alliin eluted immediately after the void volume, which corresponds to a retention time of 3.65 min. A typical high-performance liquid chromatogram of standard alliin (a), fresh garlic (b), and dried garlic extract (c) is shown in Figure 1 The alliin content in the aqueous extract of fresh garlic was observed to be more than that of the dried garlic extract. The data revealed that fresh garlic contains higher amount of alliin (0.25 μg/mg) in comparison to dried garlic (0.13 μg/mg).

Growth inhibitory activity of alliin

The antiproliferative activity of alliin in relation to gastric cancer was investigated by its effect on cell growth in human AGS cell line. The result indicated that alliin showed a marked growth inhibition in a dose-dependent manner with half maximal inhibitory concentration (IC_{50}) value very closely to 100 μM which was evidenced by the reduction of viable cells shown in Figure 2 by keeping vincristine (IC_{50}-15 μM) as a standard. Hence, the IC_{50} concentration of alliin was considered for further mechanistic studies. In the case of normal intestinal cell INT-407, alliin neither induced proliferation nor inhibited the cell growth.

AGS cells treated with alliin stained by AO/EB dual stain evaluates the nuclear morphology of apoptotic cells. Cells stained green represent viable cells, whereas yellow staining represented early apoptotic cells, and reddish or orange staining represents late apoptotic cells. In the control, uniformly green live cells with normal and large nucleus

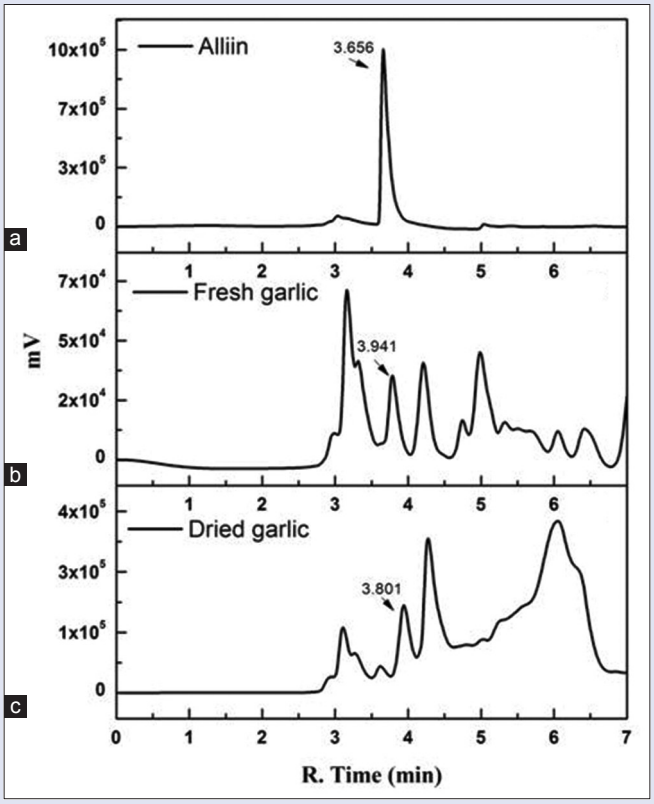

Figure 1: High-performance liquid chromatography of garlic extracts (a) high-performance liquid chromatography of standard alliin (b) high-performance liquid chromatography of dried garlic (c) high-performance liquid chromatography of fresh garlic

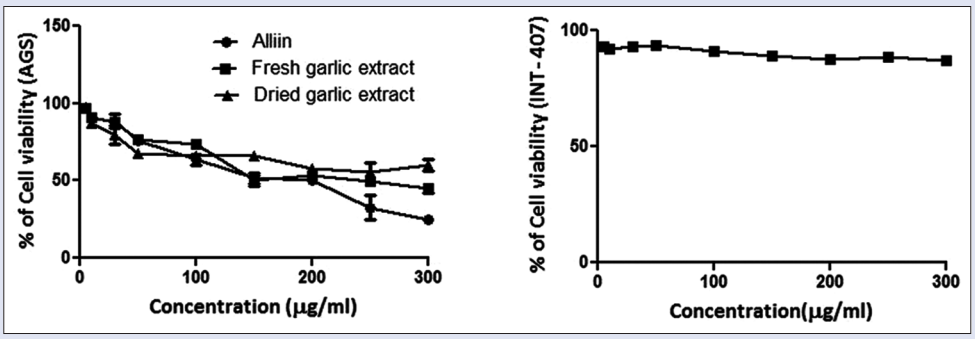

Figure 2: Cell viability potential of alliin, fresh garlic extract and dried garlic extract in gastric adenocarcinoma and normal intestinal (INT-407) cells. The data are expressed as mean ± standard error of the mean of three separate experiments

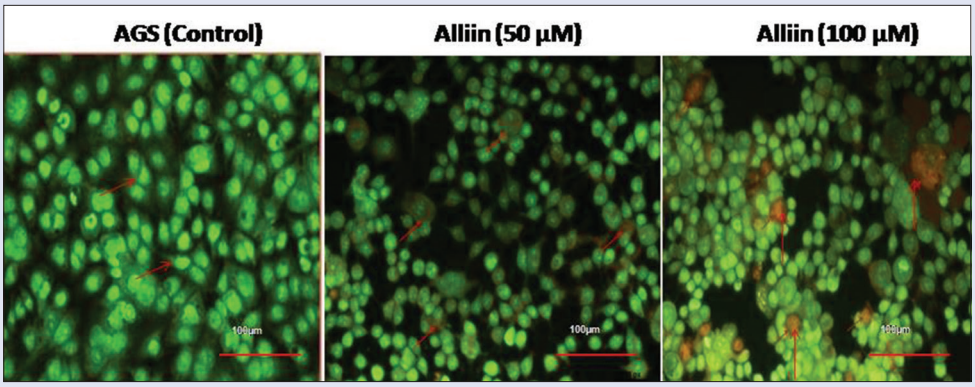

Figure 3: Morphological changes of alliin-treated cells by AO/EB staining viewed under ×20. Gastric adenocarcinoma cells without treatment show live cells gastric adenocarcinoma cells treated with 50 µM alliin show early apoptotic cells gastric adenocarcinoma cells treated with 100 µM alliin show late apoptotic cells

were observed, whereas in alliin-treated cells yellow, orange, and red staining was observed [Figure 3]. Alliin-treated cells showed significant morphological changes such as cell shrinkage and reduced cell density when compared with untreated cells. Hence, it confirms that alliin significantly induced apoptosis in gastric cancer cells.

In the present investigation, DNA fragmentation analysis was done to determine the presence of apoptosis in alliin-treated AGS cells. However, the control AGS cells had higher confluency of monolayer, and they were morphologically normal. Condensed and fragmented nuclei of AGS cells with alliin treatment at 100 and 200 µM were observed compared to untreated normal AGS cells which is shown in Figure 4. In this connection, DNA fragmentation analysis in the present investigation denotes that AGS cells treated with alliin at concentrations of 50, 100, and 200 µM resulted in DNA damage.

Quantification of apoptosis

By taking into account the growth-inhibitory effects and apoptotic event, we were interested in quantifying the number of apoptotic cells induced by alliin in AGS cells. The combination of Annexin V-FITC and PI allows the differentiation among early apoptotic cells (annexin V positive, PI negative), necrotic cells (annexin V positive, PI positive), and viable cells (annexin V negative, PI negative). Notably, in alliin-treated cells, the percentage of apoptotic cells increased from 0.01% in control cells to 56% [Figure 5]. The results of dual-color flow cytometry analysis confirmed that alliin can induce apoptosis in AGS cells in a dose-dependent manner.

To determine whether suppression of cell proliferation by alliin results from inhibition of cell cycle progression, cell cycle analysis was carried out to evaluate the distribution of actively dividing cells before the induction of apoptosis. Cell cycle analysis for both in control cells and cells treated with alliin are shown in representative histogram in Figure 6a and the respective peaks for different phases of cell cycle is shown in Figure 6b. The results shown in Figure 6a represented that alliin dose-dependently increased the S-phase population in AGS cells and a corresponding decrease of cells in G2-M phase.

Alliin-mediated reactive oxygen species generation and mitochondrial membrane depolarization (ΔΨm)

The elevations in intracellular ROS induced by alliin in AGS cells were detected by DCFDA (2,7-dichlorofluorescein diacetate) which is a cell-permeant indicator for ROS that is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. Green (C-DCDHF-DA) fluorescence intensities of alliin-treated cells shown in Figure 7 are higher than those of control cells.

Depolarization of ΔΨm results in the loss of Rhodamine-123 from the mitochondria and a decrease in intracellular fluorescence. The loss of mitochondrial membrane potential to a small extent was observed in AGS cells after exposure with increasing concentrations of alliin [Figure 8] in comparison to control cell.

Effect of alliin on apoptosis-related proteins

Changes in the level of apoptotic-related proteins (Bax, Bcl-2, caspase-9, caspase-3, and cytochrome-C) were examined to determine the

mechanism by which alliin induces apoptosis [Figure 9]. It is evidenced that alliin significantly downregulated Bcl-2 and upregulated Bax expression. Similarly, treatment of AGS cells with alliin also significantly induced the release of cytochrome C from the mitochondrial matrix which induces the caspase cascade in a dose-dependent manner.

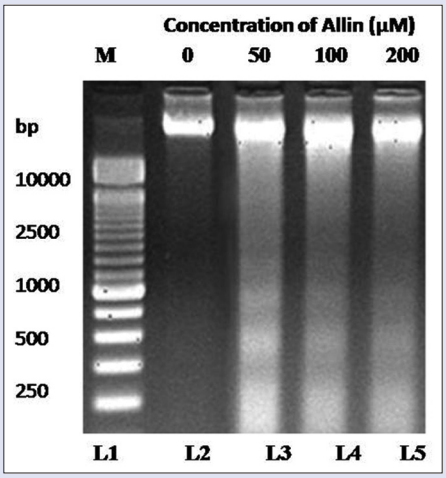

Figure 4: DNA fragmentation analysis of gastric adenocarcinoma cells with alliin treatment. Lane 1 represents MW marker of 1 kb, Lane 2 represents control cell, and Lane 3, 4, and 5 shows alliin at different concentrations (50,100, and 200 μM)

DISCUSSION

Cancer is a conglomeration of a group of pathologies which share similar characteristics in the various types of cancer. All living cells in the body as well as people of all ages and in both genders are affected by this deadly disease.[19] Gastric cancer which involves multifaceted causation comparatively affects a large number of old-aged people leading to increased mortality. Hence, identifying a possible intervention to combat the disease condition is imperative. Garlic which is a common spice used in the Indian kitchen possesses intrinsic medicinal properties such as anti-inflammatory, antioxidant, anticancer, antifungal, and antibacterial property.[20-24] A number of studies revealed that allicin a bioactive compound of garlic exhibits anticancer property.[25] However, there are very few studies on alliin, the precursor of allicin. Hence, it prompted us to study the role of alliin and identify the mechanism of action in gastric cancer.

Alliin, a natural constituent of fresh garlic and a derivative of amino acid cysteine, has been evaluated for its apoptotic potential in this study. As per the HPLC analysis, the alliin content in fresh garlic is higher when compared to dried garlic. As it has been noted that garlic enzymatically produces allicin when injured, but not all of the alliin is being converted to allicin. Levels of alliin content can vary with different processing

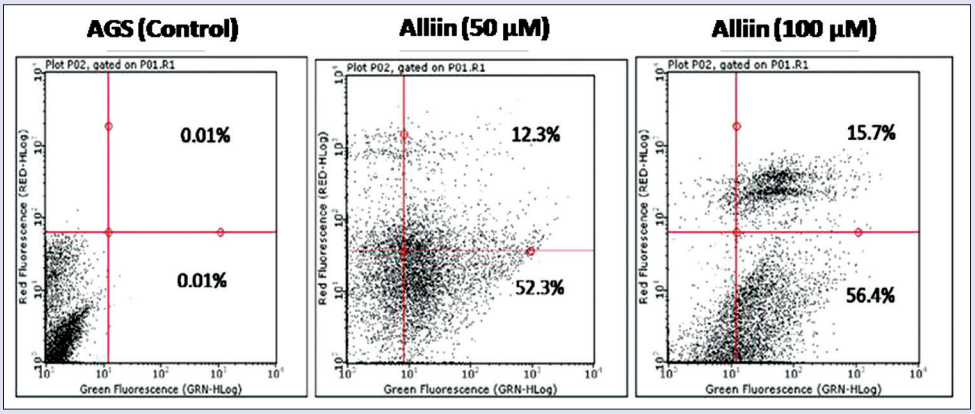

Figure 5: Apoptosis assay by annexin V-PI-based immunofluorescence. The GRN-HLog axis denotes annexin-V, and the RED-HLog axis denotes PI. The numbers indicated represent the percentage (mean ± standard deviation) of gated annexin-V+/PI – and annexin-V+/PI + cells. Results are mean of triplicate analysis

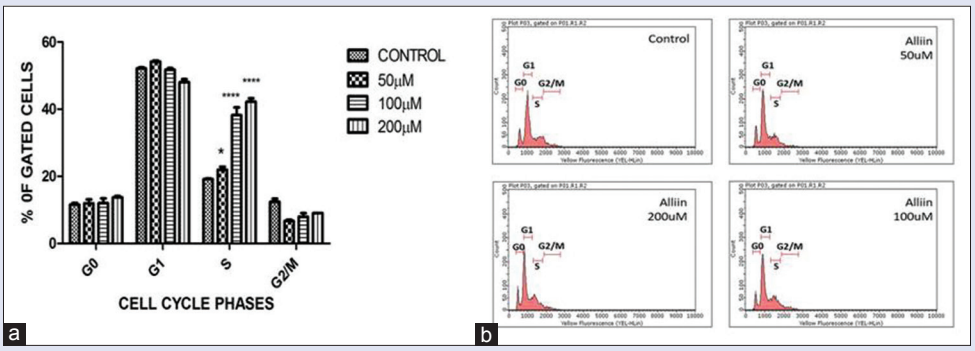

Figure 6: Effect of alliin on cell cycle distribution in gastric adenocarcinoma cells. (a) Flow cytometric histogram representing percentage of gated cells in different phases of cell cycle. (b) Respective peaks showing DNA distribution in different phases of cell cycle.*$P < 0.05$ and ****$P < 0.0001$ in alliin (50,100 and 200 μM) in comparison to control cells

conditions.[26] However, based on the results obtained in the present study on the two types of garlic, fresh garlic [Figure 1b] which contain highest content of alliin (0.25 μg/mg) is suitable for consumption, whereas dried garlic [Figure 1c] contains only (0.13 μg/mg) of alliin and shows minimal bioactivity Figure 2. Hence, it is recommended that fresh garlic may be considered as effective functional food with higher amount of alliin compared to dried garlic.

Although other phytocompounds of garlic such as diallyl sulfide, diallyl disulfide, and diallyl trisulfide have been proven for their anticancer activity,[21,27] there are very few data available characterizing alliin's anticancer activities. In this study, the effect of alliin on the proliferation of human gastric cancer and noncancer cell has been examined in detail for the first time. The present study revealed that alliin treatment decreased the percentage of viable cells [Figure 2], with a IC_{50} value of 100 μM. Neither alliin showed any proliferation nor any reduction on the growth of normal cells (INT-407) confirming its role on only cancer cells and not on normal cells.

Alliin's anticancer and antiproliferative activities probably reflect several mechanisms of action as with other types of chemopreventive agents, including nonsteroidal anti-inflammatory drugs. The present study reveals that alliin inhibits growth and induces apoptosis within 24 h of treatment which was confirmed by AO/EB staining. Further, after only 24 h of treatment, alliin prevented cells from entering the G2-M phase of the cell cycle, resulting in the accumulation of cells in the S phase. Some studies reported that different organosulfur compounds of garlic similarly block the cell cycle at the G1 or G2/M phase of both malignant and nonmalignant cells. The compounds such as ajoene and diallyl sulfide arrested the HL-60 human myeloid leukemia cells at the G2/M phase[28,29] while S-allylmercaptocysteine caused a G1 arrest in human umbilical vein endothelial cells.[30]

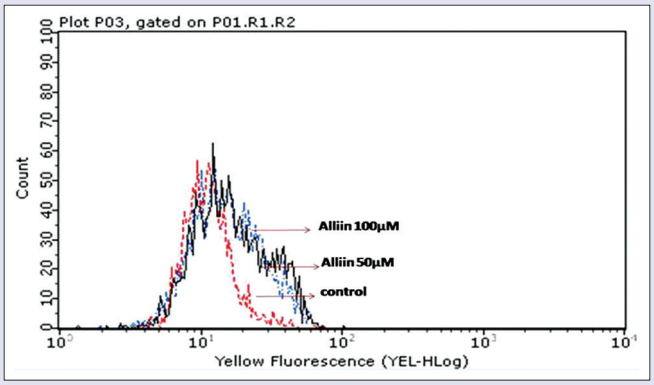

Figure 7: Reactive oxygen species generation in gastric adenocarcinoma cells. The YEL-HLog on the X-axis denotes reactive oxygen species generation and cell count on the Y-axis

Electron and proton transport through the inner membrane of mitochondria is responsible for cellular ROS generation, and mitochondria-mediated abnormal ROS is widely recognized as an aggravating factor in numerous human diseases.[31] ROS are believed to involve in several cellular functions, such as cell proliferation, differentiation, and apoptosis.[32] In this study, the intracellular ROS level in AGS cells treated with different concentration of alliin (50 and 100 μM) was measured by DCFH-DA assay. ROS in alliin-treated groups was significantly elevated as compared with the control group cells. Considering the influence of alliin treatment (for 24 h) on cell viability and apoptosis, the attenuation of ROS production may dramatically be due to cell death induced by alliin. Increase in ROS level generation leads to potentially cytotoxic oxidative stress which is identified as apoptosis inducers and modulators[33] and has been revealed to play a vital role in chemotherapy.[32] It is worth mentioning that the organosulfur compound alliin is a potential modulator of apoptosis.

Mitochondrial electron transport chain is the most important internal resource of ROS, of which NADH is the important factor. The generation of increased ROS from mitochondria causes dysfunction of the electron transport chain. It was illustrated in Figure 7 that alliin-triggered apoptosis associated with excessive ROS generation in AGS cells, demonstrating that alliin might exhibit anticancer activity by excessive generation of ROS. Typically, AGS cells treated with alliin recorded elevated ROS levels over vehicle-treated control cells. Simultaneously, after alliin treatment on AGS cells, apoptotic protein Bax was upregulated whereas Bcl-2 was downregulated and these alterations displaced the balance between pro-and anti-apoptotic family members

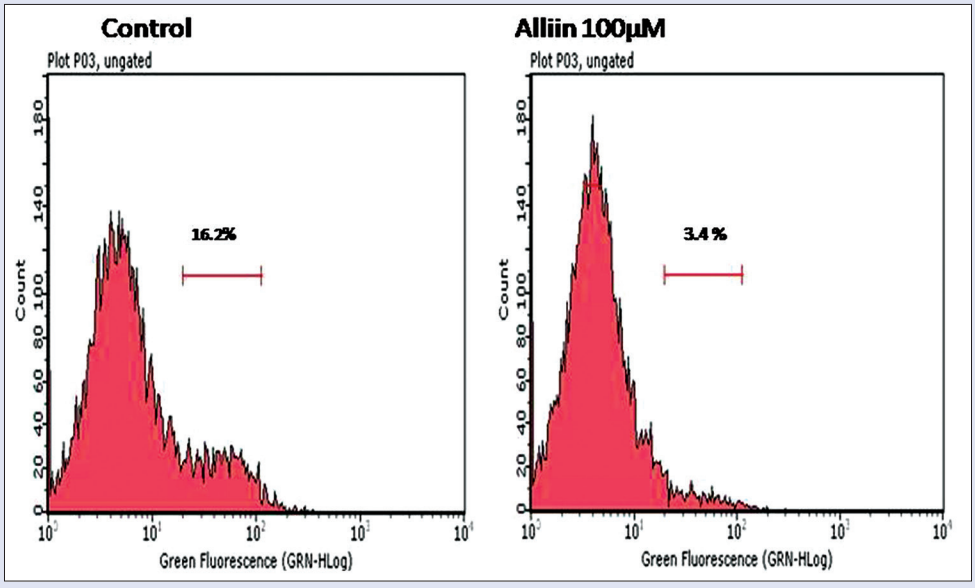

Figure 8: Mitochondrial membrane potential changes of cells treated with alliin. The GRN-HLog on the X-axis denotes mitochondrial membrane potential and cell count on the Y-axis

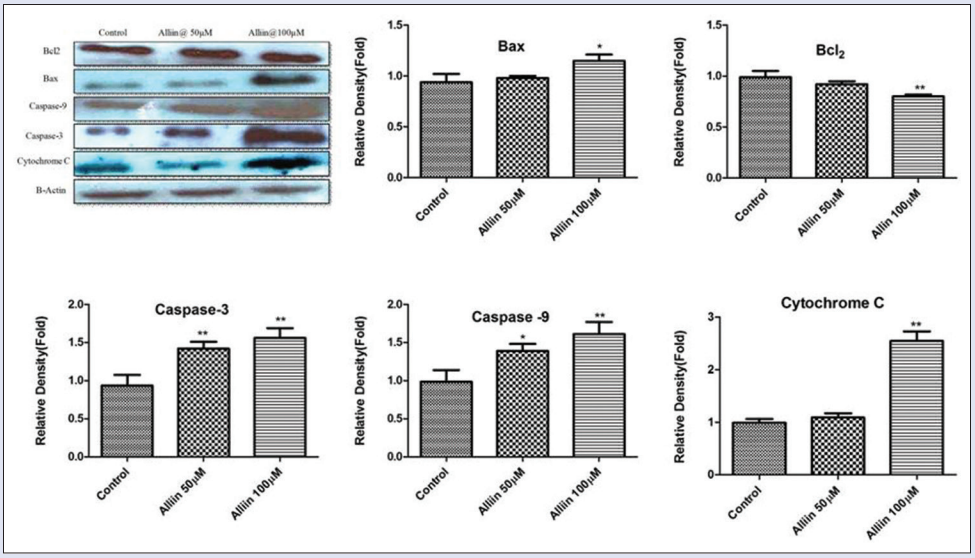

Figure 9: Effect of alliin on apoptosis-related proteins. The levels of apoptotic proteins (Bax, Bcl-2, caspase-3, caspase-9, and cytochrome C) were accordingly plotted as bar charts by keeping ß-Actin as a housekeeping gene. *$P < 0.05$ and **$P < 0.01$ in alliin (50,100 μM) compared to control cells

on mitochondrial outer membrane toward apoptosis[34,35] which caused a loss of $\Delta\Psi_m$ and outburst of cytochrome-C from the mitochondrial space into the cytoplasm. Cytochrome-C in the cytoplasm forms an apoptosome that in turn activates caspase-9 from its inactive state. The protein level of cytochrome-C, caspases-9 and -3 were found to be elevated by alliin treatment in AGS cells which confirms that caspase-9 activates procaspase-3 to execute apoptosis.

CONCLUSION

The results of the present study revealed that alliin inhibited proliferation of AGS cells through induction of apoptosis and apoptotic cell death involves both the intrinsic and extrinsic pathways. Alliin also promoted S-phase arrest in AGS cells preventing the cells to enter into G2/M phase. The data indicate that garlic a medicinal spice may be explored in detail due to its several pharmaceutical properties which can be a potentially effective therapy for stomach cancer. To the best of our knowledge, this is the first report on the anticancer activity of alliin against gastric cancer.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, *et al.* Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359-86.
2. Dikshit RP, Mathur G, Mhatre S, Yeole BB. Epidemiological review of gastric cancer in India. Indian J Med Paediatr Oncol 2011;32:3-11.
3. Yang L. Incidence and mortality of gastric cancer in China. World J Gastroenterol 2006;12:17-20.
4. Fock KM, Ang TL. Epidemiology of *Helicobacter pylori* infection and gastric cancer in Asia. J Gastroenterol Hepatol 2010;25:479-86.
5. Kaefer CM, Milner JA. The role of herbs and spices in cancer prevention. J Nutr Biochem 2008;19:347-61.
6. Qidwai W, Ashfaq T. Role of garlic usage in cardiovascular disease prevention: An evidence-based approach. Evid Based Complement Alternat Med 2013;2013:125649.
7. Yamasaki T, Teel RW, Lau BH. Effect of allixin, a phytoalexin produced by garlic, on mutagenesis, DNA-binding and metabolism of aflatoxin B1. Cancer Lett 1991;59:89-94.
8. Amagase H. Clarifying the real bioactive constituents of garlic. J Nutr 2006;136:716S-725S.
9. Amagase H, Petesch BL, Matsuura H, Kasuga S, Itakura Y. Intake of garlic and its bioactive components. J Nutr 2001;131:955S-62S.
10. Elkayam A, Mirelman D, Peleg E, Wilchek M, Miron T, Rabinkov A, *et al.* The effects of allicin on weight in fructose-induced hyperinsulinemic, hyperlipidemic, hypertensive rats. Am J Hypertens 2003;16:1053-6.
11. Ahmed N, Laverick L, Sammons J, Zhang H, Maslin DJ, Hassan HT, *et al.* Ajoene, a garlic-derived natural compound, enhances chemotherapy-induced apoptosis in human myeloid leukaemia CD34-positive resistant cells. Anticancer Res 2001;21:3519-23.
12. Siddique YH, Afzal M. Antigenotoxic effect of allicin against methyl methanesulphonate induced genotoxic damage. J Environ Biol 2005;26:547-50.
13. Dethier B, Laloux M, Hanon E, Nott K, Heuskin S, Wathelet JP, *et al.* Analysis of the diastereoisomers of alliin by HPLC. Talanta 2012;101:447-52.
14. Lee J, Gupta S, Huang JS, Jayathilaka LP, Lee BS. HPLC-MTT assay: Anticancer activity of aqueous garlic extract is from allicin. Anal Biochem 2013;436:187-9.
15. van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: The MTT assay. Methods Mol Biol 2011;731:237-45.
16. Cury-Boaventura MF, Pompéia C, Curi R. Comparative toxicity of oleic acid and linoleic acid on Jurkat cells. Clin Nutr 2004;23:721-32.
17. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. J Immunol Methods 1995;184:39-51.
18. Bild AH, Yao G, Chang JT, Wang Q, Potti A, Chasse D, *et al.* Oncogenic pathway signatures in human cancers as a guide to targeted therapies. Nature 2006;439:353-7.
19. Nair MK, Varghese C, Swaminathan R. Cancer: Current Scenario, Intervention Strategies and Projections for 2015. Burd Dis India; 2005. p. 219.
20. Dkhil MA, Abdel-Baki AS, Wunderlich F, Sies H, Al-Quraishy S. Anticoccidial and antiinflammatory activity of garlic in murine Eimeria papillata infections. Vet Parasitol 2011;175:66-72.
21. Lai KC, Kuo CL, Ho HC, Yang JS, Ma CY, Lu HF, *et al.* Diallyl sulfide, diallyl disulfide and diallyl trisulfide affect drug resistant gene expression in colo 205 human colon cancer cells *in vitro* and *in vivo*. Phytomedicine 2012;19:625-30.

22. Lanzotti V, Barile E, Antignani V, Bonanomi G, Scala F. Antifungal saponins from bulbs of garlic, *Allium sativum* L. var. Voghiera. Phytochemistry 2012;78:126-34.
23. Meriga B, Mopuri R, MuraliKrishna T. Insecticidal, antimicrobial and antioxidant activities of bulb extracts of *Allium sativum*. Asian Pac J Trop Med 2012;5:391-5.
24. Pundir RK, Jain P, Sharma C. Antimicrobial activity of ethanolic extracts of *Syzygium aromaticum* and *Allium sativum* against food associated bacteria and fungi. Ethnobotanical Leafl 2010;2010:11.
25. Hirsch K, Danilenko M, Giat J, Miron T, Rabinkov A, Wilchek M, *et al.* Effect of purified allicin, the major ingredient of freshly crushed garlic, on cancer cell proliferation. Nutr Cancer 2000;38:245-54.
26. Prati P, Henrique CM, Souza de AS, Silva da VS, Pacheco MT. Evaluation of allicin stability in processed garlic of different cultivars. Food Sci Technol 2014;34:623-8.
27. Yu CS, Huang AC, Lai KC, Huang YP, Lin MW, Yang JS, *et al.* Diallyl trisulfide induces apoptosis in human primary colorectal cancer cells. Oncol Rep 2012;28:949-54.
28. Dirsch VM, Gerbes AL, Vollmar AM. Ajoene, a compound of garlic, induces apoptosis in human promyeloleukemic cells, accompanied by generation of reactive oxygen species and activation of nuclear factor kappaB. Mol Pharmacol 1998;53:402-7.
29. Zheng S, Yang H, Zhang S, Wang X, Yu L, Lu J, *et al.* Initial study on naturally occurring products from traditional Chinese herbs and vegetables for chemoprevention. J Cell Biochem Suppl 1997;27:106-12.
30. Lee ES, Steiner M, Lin R. Thioallyl compounds: Potent inhibitors of cell proliferation. Biochim Biophys Acta 1994;1221:73-7.
31. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J, *et al.* Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39:44-84.
32. Liou GY, Storz P. Reactive oxygen species in cancer. Free Radic Res 2010;44:479-96.
33. Trachootham D, Lu W, Ogasawara MA, Nilsa RD, Huang P. Redox regulation of cell survival. Antioxid Redox Signal 2008;10:1343-74.
34. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999;13:1899-911.
35. Korsmeyer SJ. BCL-2 gene family and the regulation of programmed cell death. Cancer Res 1999;59:1693s-700s.