Abstract
Cancer of the uterine cervix represents the second most common cancer in females worldwide and is a major cause of morbidity and mortality. In 2006, 9,710 women were diagnosed with cervical cancer and 3,700 women died from the disease in U.S.A. Worldwide, morbidity and mortality rates are far higher with an incidence of 4,90,000 new cases and 2,73,000 deaths occurring in 2005, according to the World Health Organization. About 80 percent of these cases occur in developing countries. High-risk HPV infection is the primary risk factor for the development of cervical carcinoma. Over 90% of cancers of the uterine cervix contain high-risk HPV DNA.

The current hypothesis for induction of uterine cervical tumors by HPV is that during the course of viral infection, the HPV genome randomly becomes integrated into the host’s genome. If the integration of the viral genome results in disruption of the viral E2 gene, then there is a loss of its control over expression of viral oncoproteins E6 and E7. Uncontrolled expression of these two proteins is a consequence, and E6 and E7 interfere with functions of tumor suppressor proteins which control both the cell cycle and apoptosis, resulting in initiation of pre-cancerous lesions and cancer. E6 protein binds to p53 via the E6-Ap protein (a host cell ubiquitin ligase) and facilitates the degradation of p53 through the ubiquitin proteasome pathway. This dramatically decreases the half-life of the p53 tumor suppressor protein in infected cells, and p53-mediated regulation of the cell cycle is lost. In addition to the loss of p53 protein, the overexpression of E6 and E7 proteins facilitates uncontrolled proliferation of the infected cell through the activation of cellular cyclins E and A. E7 protein also interferes with the functions of the cellular tumor suppressor protein retinoblastoma (pRB). Viral E7 protein binds to pRb and inhibits the interaction of pRB with E2F and other factors associated with the cell cycle. High risk E6 and E7 proteins are usually coexpressed within infected cells. By simultaneous disruption of the functions of the functions of key cellular tumor suppressors p53 and pRb, high-risk HPV E6 and E7 proteins immortalize and transform human cells, setting the stage for cancer progression.

The standard first line treatment for invasive cervical cancer is usually cisplatin (Platinol) and 5-fluorouracil (5-FU, Adrucil, Efudex) used in combination and in addition to radiation. Recurrent and late stage cervical cancers are often treated with a combination of platinum, bleomycin, methotrexate, and 5-FU. Chemotherapy is administered
intravenously, through injection, or in pill form and side effects often accompany treatment and may be severe; which can include nausea, vomiting, diarrhea, and leukopenia (low white blood cell count). Most of these side effects are a result of drug toxicity to healthy tissues such as skin, hair follicles, and epithelial cells that line the digestive tract and patient tumors can develop resistance to these therapies as well. Hence, there is a need to develop alternate, safer, less toxic, and more specific therapies for uterine cervical cancer.

Some of the most successful currently used anticancer drugs are extracts or derivatives of compounds from plants or other natural products. These include the advance class of drugs known as taxanes (Paclitaxel, Docetaxel) that come from the yew tree (Taxus baccata), and the vinca alkaloids (Vincristine, Vinblastine, Vinorelbine) which are derivatives from the periwinkle plant (Catharanthus roseus). These compounds affect the growth of all rapidly dividing cells, whether normal or malignant, and like other drugs, are associated with significant toxicity and side effects. Millions of research dollars each year go to the discovery of new natural products with limited side effects that may be used as effective antitumor agents. Some compounds that are currently being investigated are components of dietary plants. Epidemiological observations and laboratory studies, both in cell culture and animal models have indicated anticarcinogenic potential of garlic and its constituents, which has been traditionally used for varied human ailments around the world.

Garlic (Allium sativum L.) is a widely consumed herb in foodstuffs and medicines. It has been reported to reduce chemically induced esophageal, skin, pulmonary, stomach, breast, uterine cervix, colon, mammary and lung tumor. The anticarcinogenic/antitumorgenic effects of garlic and its organosulfur compounds have been attributed not only to the modulation of the antioxidation and/or drug-metabolizing enzyme (Phase I and II) system, but also to the reduction of cell proliferation and induction of apoptosis for tumor cells. Allicin, the major component present in freshly crushed garlic, is one of the most biologically active compounds of garlic. Allicin is formed from alliin by the action of allinase and gets metabolized rapidly into diallyl sulfide, diallyl disulfide, diallyl trisulfide, ajoene, S-allylmercaptocysteine, S-allylcystcine and vinyl dithiins. Allicin is readily permeable through phospholipid membranes aiding its biological
activity, but its unstable nature raises the question whether all the effects of Allicin observed are due to Allicin itself or its metabolites. Indeed, its metabolites, S-allyl mercaptocysteine, diallyl sulfide, diallyl disulfide, diallyl trisulfide, ajoene and S-allyl cysteine have been shown to possess an anticancer activity, which varies depending on the agent and the experimental model.

Earlier studies have shown that garlic-derived OSCs can suppress growth of cancer cells of different anatomical locations in association with cell cycle arrest. Garlic-derived OSCs have also been shown to modulate a number of key elements in cellular signal transduction pathways linked to the apoptotic process. Therefore, the central aim of the current study was to investigate and evaluate the effect of Allicin, an organosulfur compound of garlic, on cervical cancer HeLa cell line and monocytes from cervical cancer patients. First of all, bearing in mind the previous reports of antiproliferative and apoptotic properties of Allicin on different cancer cell lines, various experiments were carried out to check the efficacy of Allicin to inhibit the proliferation of cervical cancer HeLa cells. In addition to this, anti-inflammatory property of Allicin was also verified by performing different experiments on monocytes isolated from the blood of cervical cancer patients.

First, we investigated the effect of Allicin on HPV 18 positive cervical cancer HeLa cell line. Our results illustrated that Allicin strongly suppressed the proliferation of HeLa cells in a dose- and time-dependent manner. Furthermore, we also observed Allicin-induced cell cycle arrest in HeLa cells at G0/G1 phase along with a sub-G0/G1 population suggesting that certain amount of cells underwent apoptosis which was further confirmed by the result of FITC-Annexin V Assay. Thus, antiproliferative action of Allicin in HeLa cells could be attributed to cell cycle arrest and apoptosis. Therefore, the above combined results demonstrated that Allicin treatment caused a significant decrease in the percentage of viable cell along with a concurrent induction of apoptosis in cervical cancer HeLa cells; however, the percentage of apoptotic cells did not coincide with the percent of viable cells. This difference in cell viability and apoptosis could be due to cell cycle arrest at G0/G1 phase induced by Allicin.

The mitochondrial apoptotic pathway (intrinsic pathway) is largely mediated through Bcl-2 family proteins, which include both proapoptotic members such as Bax, Bak and
BNIP3 that promote mitochondrial permeability and antiapoptotic members such as Bcl-2 and Bcl-xL that inhibit their effects, or inhibit the mitochondrial release of cytochrome c. Another important component is the tumor suppressor protein p53, which simultaneously suppresses Bcl-2 and activates Bax. The ratio of Bax to Bcl-2 is critical to cell survival which determines whether a cell undergo apoptosis. In response to proapoptotic signals, Bax translocates to the mitochondria where it oligomerizes into a heteromeric protein channel which releases apoptotic factors, such as cytochrome c. Cytochrome c leakage supports the formation of an apoptosome complex by binding to apoptotic protease activating factor-1 (Apaf-1), which activates the caspase-9 molecules (upon cleavage of the bound zymogen procaspases-9), which in turn activate caspase-3 to initiate apoptosis. Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear enzyme PARP, followed by DNA fragmentation. In the current study, considering the previous reports on garlic and its organosulfur compounds, we further explored the involvement of mitochondrial apoptotic pathway in Allicin-induced apoptosis in HeLa cells. Our Western blot results showed that Allicin induced mitochondrial release of cytochrome c. Moreover, Allicin treated HeLa cells also exhibited an augmented caspase-9 and -3 activities which were found to be dose dependent. Likewise, Western blot results also showed cleavage and activation of procaspase-9 and -3 which further confirmed our finding of enhanced caspase-9 and -3 activities. Pretreating HeLa cells with caspase inhibitors Z-VAD-FMK, Z-DEVD-FMK (caspase-9 inhibitor) and Z-LEHD-FMK (caspase-3 inhibitor) completely abrogated Allicin-induced caspase activation and cell proliferation. Furthermore, we also demonstrated Allicin-induced PARP cleavage in HeLa cells and cleaved product of 89 KDa was found to be evident in Western blot. Similarly, results of Western blot analysis also revealed an upregulated Bax and downregulated Bcl-2 protein expressions in Allicin-treated HeLa cells.

The mitochondrial apoptotic pathway (intrinsic pathway) is also regulated by the mitochondrial permeability transition pore (mPTP). Cyclosporin A (CsA) is an inhibitor of cyclophilin D and blocks the formation of the mPTP and prevents its opening which lead to inhibition of mitochondrial membrane depolarization and Bax translocation to mitochondria. This ultimately suppresses release of cytochrome c from mitochondria and
in turn caspase activation which play important role in apoptosis. Therefore, in the
current study, the effect of CsA pretreatment on Allicin-induced suppression of
proliferation and apoptosis of HeLa cells was also investigated. Our results showed that
CsA pretreatment of HeLa cells inhibited Allicin-induced growth suppression and cell
death, corroborating the central role of mitochondria in the apoptotic process induced by
Allicin. Furthermore, Allicin induced a cell cycle arrest at G0/G1 in HeLa cells. Upon
CsA pretreatment, the percentages of cells at G0/G1 remain unchanged. So, it can be
deduced from this result that the effect of Allicin on cell cycle arrest was independent of
its proapoptotic activity.

Glutathione is the major regulator of the thiol-disulfide redox state of living cells.
Alterations in cellular free thiols have already been described as a key event in the
generation of apoptosis. Glutathione (GSH), cysteine and accessible free sulfhydryl (-SH)
groups of various proteins and peptides are rapidly oxidized by Allicin to their respective
Allyl disulfide derivatives. In the present study, the effect of Allicin on cellular GSH
content and total free thiol (-SH) content of cytosol and mitochondria was also examined.
Our results showed that Allicin induced an initial drop in GSH levels that was followed
by a regeneration process, occurring several hours after treatment. This may be due to
decreased feedback inhibition of GSH biosynthetic enzyme such as γ-glutamylcysteine
synthetase (γ-GCS) by GSH. In addition, Allicin also caused GSH depletion
concomitantly in the cytosol and mitochondria in HeLa cells. The rate of free -SH
depletion from the cytosol and the mitochondria was similar, indicating that Allicin
penetrated the mitochondria and the cell membrane equally well and reacted with free -
SH in both compartments. Furthermore, in Buthionine sulfoximine (BSO)-pretreated
cells, there was no recovery of GSH after its Allicin-induced depletion, as BSO is a direct
inhibitor of γ-GCS. Since BSO-induced depletion of GSH levels caused neither
mitochondrial permeability transition nor apoptosis (no effect on cell survival), it is likely
that BSO treatment depletes GSH mainly from the cytosol, whereas Allicin induced
depletion of mitochondrial GSH trigger the apoptotic process.

N-acetylcysteine’s (NAC) primary function in vivo is to supply cysteine necessary for
GSH synthesis and replenishment. It can also reduce disulphide bonds in proteins and
scavenge free radicals. In this study, we also investigated the effect of NAC pretreatment
on Allicin-induced growth inhibition and apoptosis of HeLa cells. Our results showed that simultaneous treatment of HeLa cells with NAC and Allicin abrogated the antiproliferative and apoptotic activity of Allicin. Furthermore, the cell cycle arrest induced by Allicin at G0/G1 phase was also shown to be abolished by NAC. The results observed could be due to NAC's key role to provide cysteine for GSH synthesis and replenishment depleted by Allicin.

HPV oncoproteins E6 and E7 can disturb cell cycle regulation and impede apoptosis by binding to two tumor suppressor proteins, p53 and pRb, respectively which results in cellular immortalization and transformation. In the current study, we also investigated the effect of Allicin on E6, E7 and p53 expression in HeLa cells. Strikingly, our results have revealed that Allicin strongly inhibited the expression of HPV 18 E6 and E7 oncoproteins in cervical cancer HeLa cells. This downregulation of E6 and E7 expression could result in cessation of cervical cancer cell growth. Furthermore, the expression of p53 tumor suppressor protein in Allicin treated HeLa cells was also found to be upregulated. In other words, Allicin could restore the function of p53 and disrupt the E6/p53 pathway which plays a vital role in cervical carcinogenesis. Since p53 simultaneously suppresses anti-apoptotic Bcl-2 and activates apoptotic Bax, its increased expression and restoration of function could also assist in programmed cell death induced by Allicin in cervical cancer cells.

Monocytes play an important role in the initiation, development, and outcome of the immune response. Immune responses and inflammatory reactions mediated by monocytes are regulated not only positively by cellular recruitment, proliferation, and cross-talk, but also negatively by apoptosis of monocytes. So, in the present study, we further investigated the effect of Allicin on survival of monocytes isolated from cervical cancer patients. Strikingly, findings of the current study demonstrated that Allicin strongly induced apoptosis in monocytes of cervical cancer patients. Because the regulation of monocytic apoptosis has a great importance in the regulation of inflammation, this might be one of the mechanisms of Allicin's anti-inflammatory effects.

Previous studies have shown that several proinflammatory cytokines (IL-1α, IL-6, or TNF-α) stimulate proliferation of carcinoma cell lines derived from several different tissues including cervix. Thus, these proinflammatory cytokines might act as paracrine or...
autocrine growth factors in promoting malignant progression of cervical cancer. Surprisingly, results of the present study showed an augmented expression of proinflammatory cytokines TNF-α, IL-1α and IL-6 in monocytes from cervical cancer patients. Furthermore, expressions of these proinflammatory cytokines in monocytes were shown to be suppressed by Allicin treatment. So, our above results indicated that suppression of proinflammatory cytokines such as TNF-α, IL-1α and IL-6 by Allicin could alter the local microenvironment of cervical tumor leading to malignant progression.

In summary, the combined results of the present study established that Allicin, an organosulfur compound of garlic (*Allium sativum*), has a potent antiproliferative and apoptotic properties against cervical cancer HeLa cell line. Allicin-induced growth inhibitory action in HeLa cells involved the cell cycle arrest and apoptosis involved the activation of caspase-9 and -3 as well as release of cytochrome c from mitochondria, downregulation of antiapoptotic Bcl-2 and upregulation of apoptotic Bax and p53. Interestingly, Allicin also suppressed the expression of HPV E6 and E7 oncoproteins implicated in cervical carcinogenesis. In addition, Allicin also induced apoptosis and suppressed the augmented expression of proinflammatory cytokines TNF-α, IL-1α and IL-6 in monocytes of cervical cancer patients. Thus, it could be concluded that in view of the above antiproliferative, apoptotic and anti-inflammatory properties, Allicin could be a promising chemotherapeutic agent in prevention of cervical cancer.