DISCUSSION
Globally, cervical cancer takes the lives of more than 250000 women each year, particularly in under-resourced areas of low-, middle-, and high-income countries. Options for cancer control and treatment have reached a point that there are interventions for control that could be adopted for virtually every resource and demographic situation (Cain et al., 2009). Women die despite the availability of attractive control options, which means that educating policy makers, women's health professionals, as well as women themselves, must become a major focus for ongoing control of this disease. The human right to life, to prevention of suffering, and to education are all key rights linked to improving the control of cervical cancer and saving the lives of women, particularly in resource-poor parts of the world (Cain et al., 2009).

Emerging evidence demonstrates that targeting the tumor proteasome is a promising strategy for cancer therapy (Yang et al., 2009). Results about the clinical application of specific proteasome inhibitors and natural products with proteasome-inhibitory activity for cancer prevention or therapy have been reviewed (Yang et al., 2009). Bortezomib, the reversible proteasome inhibitor that first entered clinical trials, has been studied extensively as a single agent and in combination with glucocorticoids, cytotoxic agents, immunomodulatory drugs and radiation as treatment for multiple myeloma and other hematological malignancies. There is less evidence of bortezomib's efficacy in solid tumors. Novel irreversible proteasome inhibitors, NPI-0052 and carfilzomib, have also been developed and clinical trials are underway therapy (Yang et al., 2009). Natural products with proteasome-inhibitory effects, such as green tea polyphenol (-) epigallocatechin-3-gallate (EGCG), soy isoflavone genistein, and the spice turmeric compound curcumin, have been studied alone and in combination with traditional chemotheraphy and radiotherapy against various cancers. There is also interest in developing these natural compounds as potential chemopreventive agents (Yang et al., 2009). Thus, in the present study, EGCG – a natural antioxidant was employed to explore the potential chemopreventive mechanism in cervical cancer.

The major constituents of green tea are the Flavan-3-ols (catechins) that include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC). These polyphenol compounds have antioxidative and anti-mutagenic properties. A variety of antioxidants and
chemopreventive agents are cytotoxic to cancer cells. Cellular growth inhibition by green tea has been established in many tumor cells where EGCG has been a prime candidate for mediating this effect. Recently, anti-proliferative and anti-cancer action of EGCG has been reported in cancer cell lines (Ji et al., 2006; Pasckha et al., 1998; Elatter and Virji, 2000; Jung et al., 2001; Ahn et al., 2003; Huh et al., 2004). A vast variety of naturally occurring substances are known to protect against experimental carcinogenesis. It is becoming increasingly evident that certain phytochemicals, particularly those included in our daily diet, may have important cancer chemopreventive properties (Sanaha et al., 1997). Some anti-inflammatory chemopreventive agents have been found to suppress growth and proliferation of transformed or malignant cells through induction of programmed cell death or apoptosis (Bellosillo et al., 1998).

Regarding EGCG, population-based and clinical studies indicate that the antioxidant properties of green tea may help prevent atherosclerosis, particularly coronary artery disease. It has been reported that 70% EGCG is a potent tool in nutritional arsenal not only as an antioxidant, but to address arterial inflammation. Highly sensitive C-reactive protein (hs-CRP) is a marker of arterial inflammation. Inflammation is also believed to play a role in heart disease; EGCG is a potent anti-inflammatory. According to Japanese research, green tea reduces the levels of LDL or 'bad' blood cholesterol, thereby reducing the risk of coronary heart disease. Research also indicates that tea polyphenols may reduce the activity of platelets, which are the clotting agents of the blood. This is good, because 'sticky' blood is more likely to form artery-blocking clots. Green tea has demonstrated an ability to lower total cholesterol and raise HDL ("good") cholesterol in both animals and people. This suggests that polyphenols in green tea may block the intestinal absorption of cholesterol and promote its excretion from the body. EGCG has been reported to inhibit lipid peroxidation, an oxidative process implicated in several pathologic conditions, including atherosclerosis. It has been suggested that EGCG and other tea catechins suppress tumor promotion by inhibiting the release of tumor necrosis factor-alpha, which is believed to stimulate tumor promotion and progression of initiated cells as well as premalignant cells. Furthermore, EGCG was shown to reduce specific binding of both the 12-O-tetradecanoylphorbol-13-acetate (TPA)-type and the okadaic acid-type tumor promoters (the two major classes of tumor-promoting agents) to their
receptors. An EGCG acts against urokinase, an enzyme often found in large amounts in human cancers, inhibits ornithine decarboxylase (a rate-limiting enzyme closely associated with tumor promotion), and blocks type-1 5alpha-reductase (5AR). Inhibitors of 5AR may be effective in the treatment of 5 alpha dihydrotestosterone-dependent abnormalities, such as benign prostate hyperplasia, prostate cancer, and certain skin diseases. EGCG, inhibits tumor growth by inhibiting VEGF induction in human colon carcinoma cells. In the in vitro studies, (-)-epigallocatechin gallate (EGCG) inhibited Erk-1 and Erk-2 activation in a dose-dependent manner. However, other tea catechins such as (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin (EC) did not affect Erk-1 or 2 activation at a concentration of 30 M. EGCG also inhibited the increase of VEGF expression and promoter activity induced by serum starvation. In the in vivo studies, athymic BALB/c nude mice were inoculated subcutaneously with HT29 cells and treated with daily intraperitoneal injections of EC (negative control) or EGCG at 1.5 mg day mouse starting 2 days after tumour cell inoculation. Treatment with EGCG inhibited tumor growth (58%), microvessel density (30%), and tumour cell proliferation (27%) and increased tumour cell apoptosis (1.9-fold) and endothelial cell apoptosis (3-fold) relative to the control condition ($P<0.05$ for all comparisons). EGCG may exert at least part of its anticancer effect by inhibiting angiogenesis through blocking the induction of VEGF (Fatima, Z., 2008). Tea polyphenol EGCG inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines (Barnes and Cave, 2003). Hyper-methylation of CpG islands in the promoter regions is an important mechanism to silence the expression of many important genes in cancer. Treatment of human esophageal cancer KYSE 510 cells with 5-50 microM of EGCG for 12-144 h caused a concentration- and time-dependent reversal of hypermethylation of p16(INK4a), retinoic acid receptor beta (RARbeta), O(6)-methylguanine methyltransferase (MGMT), and human mutL homologue 1 (hMLH1) genes as determined by the appearance of the unmethylation-specific bands in PCR. This was accompanied by the expression of mRNA of these genes as determined by reverse transcription-PCR (Zeeshan, 2008) thereby demonstrating for the first time the inhibition of DNA methylation by a commonly consumed dietary constituent and in turn suggesting for the potential use of EGCG for the prevention or reversal of related gene silencing in the prevention of carcinogenesis. In addition to this EGCG activates endothelial nitric oxide synthase by a PI3K-, PKA-, and Akt-dependent pathway, and leads to
endothelial-dependent vasorelaxation, the EGCG-induced endothelium-dependent vasodilation is primarily based on rapid activation of eNOS. This suggests that tea catechins may reduce the risk of cardiovascular (Zeeshan, 2008). Interleukin-1beta (IL-1beta) induced inflammatory response in arthritic joints include the enhanced expression and activity of matrix metalloproteinases (MMPs) and their matrix degrading activity contribute to the irreversible loss of cartilage and may also be associated with sustained chronic inflammation. EGCG inhibited the IL-1 beta-induced mRNA and protein expression of MMP-1, and MMP-13 in human chondrocytes, a differential dose-dependent effect of EGCG on the expression and activity of MMPs and on the activities of transcription factors NF-kappaB and AP-1 and provide insights into the molecular basis of the reported anti-inflammatory effects of EGCG (Zeeshan, 2008). It's well established that fruits and vegetables contain components like polyphenols, terpenes, alkaloids, and phenolics that may provide substantial health benefits beyond basic nutrition. Research over the last decade has shown that several micronutrients in fruits and vegetables reduce cancer (Aggarwal and Shishodia, 2006). The active components of dietary phytochemicals that most often appear to be protective against cancer are curcumin, genistein, resveratrol, diallyl sulfide, S-allyl cysteine, allicin, lycopene, capsaicin, diosgenin, 6-gingerol, ellagic acid, ursolic acid, silymarin, anethol, catechins, eugenol, isoeugenol, dithiolthiones, isothiocyanates, indole-3-carbinol, isoflavones, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, Vitamin C, d-limonene, lutein, folic acid, beta carotene, selenium, Vitamin E, flavonoids, and dietary fiber. These dietary agents are believed to suppress the inflammatory processes that lead to transformation, hyperproliferation, and initiation of carcinogenesis. Their inhibitory influences may ultimately suppress the final steps of carcinogenesis as well, namely angiogenesis and metastasis (Aggarwal and Shishodia, 2006).

Various workers on cervical cancer HeLa cell lines have extensively carried out studies in multiple directions, but no or little work has been done on cervical cancer monocytes. Thus, the present study involves investigations on the effect of EGCG – a green tea polyphenol as well as a natural antioxidant on two cervical cancer model systems, which are: (a) monocytes and biopsies from cervical cancer patients and (b) cervical cancer cell lines i.e. HeLa cell lines. As the active role-played by reactive oxygen species (ROS) in the cervical cancer is well established,
thus an attempt was made in the present study to probe natural compounds having antioxidant properties in arresting ROS in cancer cells. Thus, comparative efficacy of natural antioxidants was carried out on cervical cancer monocytes versus HeLa cell lines.

The most striking finding of the present study is the model system involving monocytes and biopsies from cervical cancer patients showed high magnitude anti-inflammatory as well as apoptotic effect of EGCG (0-100 μg/ml), while on the other hand, EGCG (0-100 μg/ml) exerted high magnitude anti-inflammatory but low apoptotic effect on HeLa cell lines. Glutathione directly reacts with ROS, and GPx catalyzes the removal of hydrogen peroxide (Yokoigawa et al., 2005). Decrease in GPx activity indicates impairment of hydrogen peroxide-neutralizing mechanisms (Ottaviano et al., 2006). Co-culturing of cancerous (cervical) cells with NAC, turmeric or EGCG seems to induce apoptosis or acted as anti-inflammatory. Furthermore, apart from the above, a decline in GPx activity was observed in cervical cancer monocytes and cervical HeLa cells that were untreated or treated with H2O2 thereby correlating with earlier reports that substantial amounts of ROS are being generated in cancerous cells due to cellular activation (Islam et al., 2004). Enhancement / amelioration of GPx activity in cervical cancer monocytes and cervical HeLa cell cultures after addition of NAC, a precursor of in vivo antioxidant glutathione, indicates reversal of impaired neutralizing mechanisms. Surprisingly, here augmented amelioration in GPx activity was observed when EGCG was co-cultured instead of NAC, indicating EGCG to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in cancerous cell. When compared to healthy monocytes, cervical cancer monocytes as well as HeLa cell lines exhibited an appreciable EGCG-mediated suppression by around 2.14-folds and 1.98-folds respectively in the GPx activity. On the contrary, upon co-culturing of cervical cancer monocytes and HeLa cell lines with varying doses of EGCG, an appreciable amelioration in the GPx activity was recorded. Interestingly, EGCG was found to possess a higher potential to ameliorate the GPx activity in cervical cancer monocytes than in HeLa cell lines. Similar were the observations recorded with NAC and turmeric. Furthermore, the present study shows enhanced GPx activity by EGCG, which correlated inversely with the downregulation of TNF-alpha, IL-1 and IL-6 protein expressions and ROS in cervical cancerous cells. As suggested by (Aggarwal
and Shishodia, 2006) as well as by the present study, since, currently available modern medicines for treating cancers are very expensive, toxic, and less effective in treating the disease, thus, one must investigate further in detail the agents derived from natural sources, described traditionally, for the prevention and treatment of cancer and disease. Voluminous clinical trials are needed to validate the usefulness of these agents either alone or in combination with existing therapy.

Although the chemopreventive effect of EGCG has been extensively studied by a number of workers in cancerous cell lines (Barnes and Cave, 2003; Ahn et al., 2003; Wu et al., 2009) but its apoptotic effect and mechanism in biopsies and monocytes of cervical cancer patients still remains to be properly understood. Thus, in the present study, we show the mechanism of EGCG mediated apoptosis in monocytes of cervical cancer patients which involved activation of caspase-3, the executor of apoptosis and cleavage of PARP - a specific marker of apoptosis as well as suppression of IL-6. EGCG was highly effective in inhibiting the viability of cervical cancer monocytes by PARP cleavage in response to activation of caspase-3. This resulted in the loss of normal PARP function which irreversibly commits the cell to die (Bellosillo et al., 1998; Rukmini et al., 2004; Islam et al., 2000; Islam et al., 2002).

Apoptosis is one of the most investigated areas in carcinogenesis because of its wide-ranging implications and possible role in therapeutic interventions (Khan et al., 2006; Nihal et al., 2005). Caspases are recognized as key molecules in apoptotic response and are reflective of the protein alterations as consequence of apoptotic changes in cell. Apoptosis occurs via two major different activation pathways (Singh et al., 2002; Raff, 1998). One pathway involves changes in mitochondrial transmembrane potential, leading to the release of cytochrome c. Cytochrome c then binds the apoptosis activating factor 1 and procaspase-9, resulting in the activation of caspase-9 by proteolytic cleavage. The other pathway starts with death receptor ligation or Fas/FasL interaction, followed by oligomerization of the receptor, recruitment of Fas-associated death domain protein (FADD), and activation of caspase-8 (Gastman, 2001). Both caspase-9 and caspase-8 are defined as initiator caspases and can in turn activate caspase-3, the executor of apoptosis (Green, 1998; Green and Reed, 1998). Also, cross communication exists between the two pathways,
as caspase-8 may activate caspase-9 via Bid, a member of Bcl-2 family (Ashkenazi and Dixit, 1998).

Our present study demonstrated the activities of caspase-3, caspase-8 and caspase-9 in cervical cancer monocytes were activated by EGCG, indicating that both death receptor-related apoptotic pathway and the mitochondria-related pathway were activated. On the contrary, suppression or reduction in cell viability was inhibited by inhibitors of caspase-3, 8 and 9 namely Z-VAD-FMK, Z-IETD-FMK and Z-LEHD-FMK respectively, which specifically blocked PARP cleavage.

We further observed that either specific caspase-8 or caspase-3 inhibitor blocked the EGCG-induced apoptosis by an appreciably high magnitude, whereas the caspase-9 inhibitor only partially inhibited it. This implies that apoptosis induced by EGCG in cervical cancer monocytes were initiated by a pathway involving the activation of caspase-8, leading to the activation of caspase-3 and the cleavage of PARP. The activation of caspase-9 is probably a secondary consequence of the activation of caspase-8 through the cross communication between the two apoptotic pathways (Islam et al., 2004; Stennicke, and Salvesen, 1998; Stennicke et al., 1998).

Cytosmears from non-neoplastic ectocervical biopsy cultures with EGCG also showed only keratinization promoting effects of EGCG and no apoptotic cells could be seen. On the contrary, cytosmears from cervical cancer biopsy cultured with EGCG showed distinguished nuclear condensation and rounded contours with deep eosinophilia of cell cytoplasm, thereby indicating for EGCG-induced apoptotic. Histopathological sections from EGCG treated test samples also showed evidences of apoptosis, showing one apoptotic cell per 2-3 high power fields to more than one dispersed apoptotic cells in a single high power field. Occasionally, apoptotic cells were seen in groups implying foci of apoptotic changes.

Next, we also report suppression of high levels of IL-6 in cultures of cervical cancer monocytes by EGCG to be dose-dependent. In accordance with its potential role in cancer progression, various types of cancer show elevated level of IL-6 (Orth et al., 1996) and that, the strong ability of malignant cells to escape immune surveillance is a well known phenomenon. The anti-tumor immune response has been
reported to be regulated by several factors, which includes cytokines produced by
tumor and other cells of tumor stroma. It seems likely that the local cytokine
microenvironment, acting on tumor cell or on the adjacent cells, can either block or
facilitate tumor growth, and that proinflammatory cytokines strongly influence the
immunologic state (Orth et al., 1996).

Treatment with EGCG increased the proportion of cells in the G1 phase of the cell
cycle and induced apoptosis. The results in the present study indicated EGCG to
decrease the anti-apoptotic Bcl-2 protein expression, and activation of caspase-3, 8
and 9, suggesting that EGCG induces apoptosis via a both cellular as well as
mitochondrial pathway, although the major pathway was cellular. Reports indicate
that treatment with EGCG inhibited phosphorylation of the EGFR, signal transducer
and activator of transcription3 (Stat3), and extracellular regulated kinase (ERK)
proteins and also inhibited basal and transforming growth factor-α-stimulated c-fos
and cyclin D1 promoter activity. EGCG at 0.1 μg/ml (a concentration found in serum
after oral administration) markedly enhanced the growth-inhibitory effects of 5-
fluorouracil. Taken together, the findings of the present study as well as by other
workers (Liao et al., 2004; Schuller et al., 2004; Lu et al., 2006) provide insights into
molecular mechanisms of growth inhibition by EGCG. Furthermore, reports also
indicate that EGCG inhibited growth and induced apoptosis in human prostate, lung,
colon, and gastric carcinoma and human leukemia cancer cell lines (Yang et al., 2005;
Ju et al., 2005; Siddiqui et al., 2006; Roomi et al., 2005). Studies with EGCG in some
cell types indicated that it could inhibit signaling pathways related to the activation
of growth factor receptors. HNSCCs often display up-regulation of TGF-α/EGFR signal
transduction pathways, (Stat3 lies downstream of the TGF-α/EGFR signaling
pathway, and Stat3 is strongly implicated in the growth of these carcinomas and in
protecting them from apoptosis.

The observations made in the present study demonstrate that EGCG, a major
component of green tea, possesses cervical cancer cell growth inhibitory properties. It
is likely that EGCG inhibits cancer cell growth through many different regulatory
pathways, along with apoptosis and cell cycle arrest. The results of the present study
are consistent with EGCG inhibiting cell growth and inducing apoptosis in cervical
cancer biopsies and HeLa cell lines. The cytotoxic properties of EGCG seemed to be
more effective against cervical cancer monocytes than HeLa cells. At this time, there
is no explanation for these differences and more experiments are needed for further analysis. The induction of apoptotic cell death by EGCG was accompanied by characteristic morphological and structural changes as well as internucleosomal DNA degradation. PARP-cleavage and FITC-Annexin assays in the present study clearly confirmed apoptosis induction in EGCG-treated cervical cancer cells. As per the manual supplied by the manufacturer of FITC-Annexin assay kit, apoptosis is a normal physiologic process, which occurs during embryonic development as well as in maintenance of tissue homeostasis. Certain morphologic features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA, characterize the apoptotic program. Loss of plasma membrane is one of the earliest features. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca²⁺ dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including FITC. This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. FITC Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) or 7-Amino-Actinomycin (7-AAD) to allow to identify early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. For example, cells that are considered viable are FITC Annexin V and PI negative; cells that are in early apoptosis are FITC Annexin V positive and PI negative; and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive. This assay does not distinguish between cells that have undergone apoptotic death versus those that have died as a result of a necrotic pathway because in either case, the dead cells will stain with both FITC Annexin V and PI. However, when apoptosis is measured over time, cells can be often tracked from FITC Annexin V and PI negative (viable, or no measurable apoptosis), to FITC Annexin V positive.
and PI negative (early apoptosis, membrane integrity is present) and finally to FITC Annexin V and PI positive (end stage apoptosis and death). The movement of cells through these three stages suggests apoptosis. In contrast, a single observation indicating that cells are both FITC Annexin V and PI positive, in of itself, reveals less information about the process by which the cells underwent their demise. Apoptosis is a tightly regulated process which involves changes in the expression of distinct genes. One group of genes regulating apoptosis are cytoplasmic aspartate specific cysteine proteases of the ICE/CED-3 family, better known as caspases (Klaus et al, 1998). Caspase-3, for example, inactivates poly (ADP-ribosyl) polymerase by proteolytically degrading the 116 kDa enzyme into an 85 kDa fragment (Duriez and Shah, 1997; Li and Darzynkiewicz, 2000; Nigata, 2000). When cervical cancer monocytes were treated with EGCG, the expression level of procaspase-3 was decreased (data not shown) indicating that the active form of caspase-3 was generated. It is therefore inferred that EGCG-induced apoptotic death in cervical cancer monocytes was mediated in part by activation of caspase-3.

In summary, EGCG, a natural antioxidant, is a potent inducer of apoptosis in monocytes, biopsies of cervical cancer patients as well as HeLa cell lines, and that, induction of EGCG-mediated apoptosis involved activation of caspase-3 / CPP32, caspase-8 and caspase-9 as well as IL-6 suppression. The apoptotic as well as anti-inflammatory / anti-proliferative effects of EGCG was more prominent in cervical cancer monocytes than in HeLa cell lines. Why EGCG is more effective in cervical cancer monocytes than in HeLa cell lines warrants further in-depth investigation. It is hoped that in view of the above aspects, whether EGCG can be promising candidate for the chemoprevention of cervical cancer might be an interesting topic for further investigation.