5.1. Effect of [6]-shogaol induced oxidative stress mediated mitochondrial dependant apoptotis in Hep-2 cells

Introduction

Squamous cell carcinoma of larynx is sixth most common class of head and neck cancers. It is primarily associated with tobacco smoking, betel quid chewing, regular consumption of alcohol. American Cancer Society (ACS) was estimated 13,560 new cases of laryngeal cancer (10,720 in men and 2,840 in women) in the United States since 2015. In India, the estimated statistics for larynx cancer the year 2015 for males 27,060 and females 4,401 which by the year 2020 will ascend to for 29,089 and 4,796 cases (Takiar et al., 2010). Globally, we have several advanced therapeutics for larynx cancer includes surgery, radiotherapy and chemotherapy, which are frequently associated with an unexcitable side effects. It affects normal cellular function and significantly reduced quality of life (McMullen and Smith, 2015). Recently, medicinal practices have several innovative chemotherapeutic agents which have played a crucial role in the treatment of cancer, but it has a poor survival rate, not improved significantly. The screen of new novel therapeutic drug has been used to competing against malignant neoplasm behavior of cancer is important to prognosis of treatment (Wang et al., 2012).

Development and progression of cancer associated with a series of events, including dysregulation of tumor suppressor gene, cellular differentiation, excessive proliferation and dysfunction of apoptotic genes (Hanahan and Weinberg, 2011). Several studies focused that apoptotic markers Bax, Bcl-2, Cytochrome-c and Caspases have emerged as novel regulating mechanisms of tumourigenesis and cell proliferation (Agca et al., 2012; Liu et al., 2012). Apoptosis is a process of programmed cell death, is characterized by unique morphological features, including alteration of mitochondria matrix, cellular fragmentation, cell shrinkage, membrane blebbing, chromatin condensation which leads to the formation of apoptotic bodies (Reed, 2000).
Regulation of pro apoptotic genes (Bax, Bid, Bak, caspases) and anti-apoptotic genes (Bcl-2, Bcl-XL, Mcl-1, Bcl-w) would be initiated apoptosis in cancer (Wong, 2011). Several investigations evidenced that naturally occurring secondary metabolites have different structure and specificity, which induce apoptosis via, induction of ROS, alteration of apoptotic signals (Pan et al., 2008; Gan et al., 2011). In this study, we explored that [6]-shogaol induces oxidative stress mediated apoptosis in Hep-2 cells.

5.2. Results

5.2.1. Cytotoxic effect of [6]-shogaol on Hep-2 cells

Fig. 5.1 shows the cytotoxic effect of [6]-shogaol on Hep-2 cells was determined by MTT assay. Cells were treated with different concentrations of [6]-shogaol (5-50 µM) for 24 h incubation, which revealed a dose-dependent inhibition of cell proliferation. Maximum cell death was observed at 50 µM concentration. Hence, the inhibitory concentration 50 (IC_{50}) apparent from growth inhibition curve, we selected 5, 10 and 20 µM doses of [6]-shogaol for further studies.

Fig. 5.1. The cytotoxicity effect of [6]-shogaol on Hep-2 cells by MTT assay. The statistical analysis was carried out using one way ANOVA. Values are represents mean ± S.D. of three experiments. \( P < 0.05 \) was significantly different from control sample.
5.2.2. Effect of [6]-shogaol on generation of intracellular ROS in Hep-2 cells.

The intracellular ROS generation was measured by DCFH-DA staining. Fig. 5.2 illustrates the levels of ROS generation in control and [6]-shogaol treated cells. Hep-2 cells were treated with different concentration of [6]-shogaol (5, 10 and 20 μM) shows significantly increased levels of ROS generation which indicated by green fluorescence as compared to untreated control cells.

Fig. 5.2. Effect of [6]-shogaol on intracellular ROS generation was evaluated with Hep-2 cells by using DCFH-DA staining. Fig. 5.2A photo micrographic image shows control and treated Hep-2 cells. Untreated Hep-2 cells show weak fluorescence DCF (i). [6]-shogaol (5, 10 and 20 μM) treated Hep-2 cell shows increased ROS generation was indicate deep DCF fluorescence intensity (ii-iv). Fig. 5.2B depiction percentage of ROS was detected by spectrofluorometer. All experiments were performed in triplicate and all the values were expressed as mean ± SD. Statistical significance was determined by a one way ANOVA followed DMRT. Asterisks indicate significant different from control (P < 0.05).
5.2.3. Effect of [6]-shogaol on lipid-peroxidation and antioxidant status

Increased the level of lipid peroxidation and depletion of antioxidants are well known biomarker of oxidative stress. We observed increased level of lipid peroxidative marker (TBARS) and decreased activities/levels of antioxidant enzymes (SOD, CAT and GSH) in [6]-shogaol treated Hep-2 cells as compared to control cells in a concentration dependent manner (Fig. 5.3).

![Graphs showing TBARS, SOD, CAT, and GPx levels](image)

Fig. 5.3. Effect of [6]-shogaol on lipid peroxidation and cellular antioxidant status in Hep-2 cells. *Enzyme concentration required for 50% inhibition of nitroblue tetrazolium reduction in one minute. **µmol of hydrogen peroxide consumed per minute. ***µg of glutathione consumed per minute. Values are given as mean ± S.D. of six experiments in each group. Values not sharing superscripts (a, b, c, d) differ significantly at $P<0.05$. vs. control (One way ANOVA followed DMRT).
5.2.4. Effect of [6]-shogaol on Mitochondrial membrane potential (MMP)

Early stage of apoptosis is trigger by alteration of mitochondrial membrane potential were assessed by lipophillic cationic dye, Rho-123 (Fig.5.4). The control cells emit high indensity of green fluorescence indicating polarized mitochondria membrane (Fig.5.4Ai). Conversely, [6]-shogaol treated Hep-2 cells were showed significant alteration of ΔΨM was often diminished green fluorescence shows in Fig.5.4Aii-iv.

Fig. 5.4. Effect of [6]-shogaol on mitochondria membrane potential (ΔΨM) was evaluated with Hep-2 cells by Rh-123 staining. (Ai) Untreated Hep-2 cells shows high fluorescence indicate polarized mitochondria membrane. Fig. 5.4(Aii-iv) shows Hep-2 cells were treated with different concentration of [6]-shogaol (5, 10 and 20 µM) for 24 h and fluorescence intensity was decreased as indicate depolarized mitochondria matrix. Fig. 5.4B depicted fluorescence intensity was detected by spectrofluorometer. All experiments were performed in triplicate and all values were expressed as mean ± SD. Statistical significance was determined by a one way ANOVA followed DMRT. The (a, b, c, d) asterisks indicate significant different from control (P < 0.05).
5.2.5. Effect of [6]-shogaol on apoptotic morphological changes in Hep-2 cells.

Fig. 5.5 shows the microscopic image shows the characteristic features of apoptosis hallmark on [6]-shogaol treated Hep-2 cells stained with AO/EtBr. The red fluorescence dye of EtBr was selectively penetrated into nuclei of apoptotic cells, while the AO (green) had only taken up healthy cells. In our results shows untreated control cells had highly green fluorescence nucleus indicate live cells (Fig.5.5Ai). Cells were treated with different concentration 5, 10 and 20 µM of [6]-shogaol showed orange color point out early apoptotic and red stained fragmented nuclei indicates late apoptosis at concentration depended manner for 24 h represented in (Fig.5.5Aii-iv).

Fig. 5.5. Illustrated fluorescence microscopic images of apoptotic morphology by dual staining (AO/EtBr). Fig. 5.5A(i) untreated Hep-2 cells, Fig.5.5A(ii-iv) shows different concentration of [6]-shogaol (5, 10 and 20 µM) treated Hep-2 cells shows increased % of apoptotic cells in concentration dependent manner. Fig.5.5B shows percentage apoptotic cells were calculated. All experiments were performed in triplicate and all values were expressed as mean ± SD. Statistical significance was determined by a one way ANOVA followed DMRT. The (a, b, c, d) asterisks indicate significant different from control (P < 0.05).
5.2.6. Effect of [6]-shogaol on nuclear damages

The nuclear damages were predicted by AO staining and Hoechst are represented in Fig. 5.6. Control cells show normal compact nuclei with deep green color Fig. 5.6a (i). Cells were treated different concentration (5, 10 and 20 µM) of [6]-shogaol shows chromatin condensation, nuclear fragmentation, and apoptotic bodies were indicated in Fig. 5.6a (ii-iv). Furthermore, the nuclear damages were observed by Hoechst staining in untreated control and [6]-shogaol treated Hep-2 cells (Fig. 5.6b).

![Fig. 5.6. Shows apoptotic nuclear fragmentation was observed by acridine orange and Hoechst staining. (a) Acridine orange staining, (b) Hoechst staining. (i) untreated control cells, (ii-iv) shows different concentration (10, 20 and 30 µM) of [6]-shogaol treated Hep-2 cells. Red and yellow arrows indicate cells nuclear fragmentation and membrane blebbing.](image-url)
5.2.7. Effect of [6]-shogaol on DNA damage

Photomicrograph image of DNA damage (comet) on control and [6]-shogaol treated cells were depicted in Fig. 5.7A. The untreated control cells shows compact circle nuclei Fig. 5.7(i). Hep-2 cells were treated with different concentration of [6]-shogaol (5, 10 and 20 µM) shows significantly increased DNA damage which appeared as comet [Fig. 5.7(ii-iv)]. The graphical observation of DNA damage (% head DNA, tail length, tail moment, Olive tail moment) in control and [6]-shogaol treated cells depicted in Fig. 5.7B.

Fig. 5.7. Effect of [6]-shogaol on DNA damage in Hep-2 cells. (A) The fluorescence microscopic image of comets cells (20X) due to DNA damage observed comet attributes. (i) Untreated control cells; (i-iv) comet figure of cells treated with different concentration of [6]-shogaol (5, 10 and 20 µM). Fig. 5.7(B) Shows DNA damage as characterized by the % head DNA, tail DNA, tail movement and olive tail movement in [6]-shogaol treated cells. All experiments were performed in triplicate and all values were expressed as mean ± SD in each group (One way ANOVA followed DMRT). The bars not sharing the common superscripts significantly different from control \( P < 0.05 \).
5.2.8. Effect of [6]-shogaol on apoptotic markers

After [6]-shogaol (5, 10 and 20 µM) treated Hep-2 cells shows down regulate the levels of Bcl-2 expression and up regulation of Bax, Cytochrome-c, Caspase-9 and Caspase-3 expression were observed at concentration depended manner. On the contrary, untreated control cells showed increased anti-apoptotic protein Bcl-2 and decreased expression of Bax, Cytochrome-c, Caspase-9 and Caspase-3 were depicted in Fig. 5.8(A). The representative graph shows the fold changes in protein expression Fig. 5.8(B). These results suggested that the activation of Bax, capase and inhibition of Bcl-2 leads to release Cytochrome-c may be one of the mechanisms that contributed to the activation of mitochondrial mediated apoptosis during [6]-shogaol treated Hep-2 cells.

![Western Blot Images](image)

Fig.5.8. Effects of [6]-shogaol on apoptotic protein Bax, Bcl-2, Cytochrome c, Caspase-9 and Caspase-3 in Hep-2 cells. (A) Shows the band intensities were quantified by Image-studio software (LI COR, USA.) and normalized to respective β-actin loading control. (B) The representative graph shows the relative protein expression of fold changes in western blots. Values are expressed as mean ± SD for three experiments. Values that do not share a common superscript letter (a, b, c) between groups differ significantly at $p < 0.05$ (One way ANOVA followed by DMRT).
5.3. Discussion

Phytochemicals are attracted among the scientific community for development of chemotherapeutic drug. Epidemiological studies suggest that a regular consumption of naturally occurring phytonutrients minimize the incidence of cancer progression for example 60% of anticancer drugs from natural origin (Wang et al., 2005; Wang et al., 2012; Russo et al., 2010; Weng and Yen, 2012). Previous studies have been reported the phenolic compounds such as quercetin (Lin et al., 2008), resveratrol (Woo et al., 2004), curcumin (Woo et al., 2005) as well as gingerols and shogaols (Ishiguro et al., 2007; Tchombe et al., 2012), exhibits anticancer effect against various cancers cell line. In this study, we examined, [6]-shogaol inhibits cell proliferation and induction of apoptosis activity against Hep-2 cells. Our results clearly indicates that [6]-shogaol effectively inhibits cell proliferation at a concentration of 20 µM (IC\textsubscript{50}) for 24 h by MTT assay (Fig.5.1). Previous studies reported that [6]-shogaol inhibits cell proliferation in various cancer cells such as ovarian, breast, lung, colon, and skin cancers (Kim et al., 2008; Rhode et al., 2007; Sang et al., 2009; Tan et al., 2013). Consistent with these evidences, our results also show significantly more potent anti-proliferative effects of [6]-shogaol.

Oxidative stress is an asymmetry between the generation and/or elimination of reactive oxygen species, cause extreme oxidative damage to macromolecules. Abundant generation free radicals induce lipid peroxidation, which are the major consequences of oxidative stress (Simon et al., 2000). Depletion of endogenous antioxidants and increase lipid peroxidation by generation of free radicals, thus may induce early stage of apoptosis in cancer (Shilpa et al., 2012; Karthikeyan et al., 2015). In our study, we observed increased ROS levels and decreased antioxidant status in [6]-shogaol treated cells at a concentration dependant manner (Fig. 5.2 and Fig. 5.3). This finding demonstrates the [6]-shogaol act as pro-oxidant, since, cancer cells possess centrally acidic region. Similarly, most of the phenolic agents act as pro-oxidant in cancer cells (Karthikeyan et al., 20011; Zheng et al., 2008; Cordero et al., 2012).
Several phenolic agents induce cellular ROS generation and oxidative stress as a gambit of preferentially killing cancer cells (Pelciano et al., 2004; Weng, and Yen, 2012). Therefore, we suggested that prooxitant role of [6]-shogaol induce intracellular ROS generation thereby depletion of antioxidant status in Hep-2 cells.

Apoptotic cell death is characterized by several hallmarks such as plasma membrane blebbing, DNA fragmentation, chromatin condensation which make out apoptotic bodies (Selvaraj et al., 2013). High concentrations of ROS are toxic to cells and it has many biochemical changes, such as oxidative DNA damage typically are modifications of purine and pyrimidine bases, its form single strand breaks (SSB) and double strand (DSB) DNA breaks (Kryston et al., 2011). Previous studies documented that dietary agent induced oxidative DNA damage in SNU-1 human gastric cancer cells (Chiang et al., 2011). In our study, oxidative DNA damage and nuclear fragmentation were analyzed in [6]-shogaol (5, 10 and 20 µM) treated Hep-2 cells. We observed increase DNA damage (% DNA fragmentation, % tail movement, % OTM) and increase nuclear fragmentation in [6]-shogaol treatment at concentration depended manner (Fig.5.7). This finding strongly support with earlier reports, [6]-shogaol induce oxidative stress, it leads to oxidative DNA damage in various carcinoma cell lines (Pan et al., 2008b; Hu et al., 2012; Ha et al., 2012).

Apoptosis is cell-intrinsic machinery, helps to eliminate potentially harmfull cells, which was mainly regulated by Bcl-2 family proteins. The deregulation of Bax/ Bcl-2 ratio results in lock of apoptosis in cancer (Renault et al., 2013). Over generation of ROS can alter the mitochondrial membrane resulting in release of Cytochrome-c from the intermembrane space into the cytosol thereby activate apoptosis-inducing factor; results in apoptosis (Ly et al., 2003; Fulda, 2010). In accordance, [6]-shogaol induced apoptosis was accompanied by alter mitochondrial membrane potential, inhibit Bcl-2 and activation of Bax, Cytochrome-c, Caspase-9 and Caspase-3 which leads to induction of mitochondrial dependent apoptosis in Hep-2 cells.
Previous studies demonstrated that [6]-shogaol induces apoptosis through activation of GADD 153 gene is highly responsive to endoplasmic reticulum (ER) stress results in decrease in levels of $\Delta \Psi M$ and activation of apoptotic gene in COLO 205 cells (Pan et al., 2008b). Similar structural molecule [8]-shogaol induced apoptosis via generation of ROS, and activation of Caspase in human leukemia cells was reported by Shieh et al. (2010). Another study also shows that ginger and structurally related compounds [6]-paradol induce apoptosis by activation Bax and Caspases in cancer cells (Elkady, 2012; Keuma et al., 2002). Despite antitumor properties of [6]-shogaol have a double bond on the carbon side chain (C=C) forming $\alpha, \beta$-unsaturated ketone moiety. These structural specificities cause apoptosis, via microtubule damage in cancer cell (Ishiguro et al., 2008; Yagihashi et al., 2008). This might be a reason for induction of apoptosis induced by [6]-shogaol in Hep-2 cells.

![Diagram](image)

Fig. 5.9. Schematic drawing proposed molecular mechanism and the overall possible signaling pathways for [6]-shogaol-induced mitochondrial dependant apoptosis in Hep-2 cells.