CHAPTER 5
DISCUSSION

Even though a large number of naturally derived metabolites are now FDA approved, however, an equal number of such products are yet to be recognized. This may include products derived from *G. lucidum*. Fungal parts, especially, basidiocarp, spore, and mycelia, contains several bioactive compounds, including polysaccharides, triterpenes, alkaloids, steroids, fatty acids, GAs, proteins/peptides, nucleotides, beta-glucans, minerals, vitamins, and fibers, etc. with incredibly versatile medicinal potential (Yuen *et al.*, 2005). As a medicinal mushroom, it lends its features in modulating several signaling pathways cardinal in various anomalies (Wasser *et al.*, 1999). *G. lucidum*, generally grown as a saprophyte on dead organic matter, but can also be propagated as root and trunk parasite of many trees and plants. It exploits the host machinery to fulfil its requirements of food and nutrition (Bagchee *et al.*, 1950). An environmentally satiated condition favors the growth and development of *G. lucidum,* whereas stress conditions tend to trim down these processes, even culminating in death. *G. lucidum*, as a parasite, depends on the host plants to obtain the nutrient resources required for survival. Hence, phenomenon such as parasitic-association, host-dependence, and resource exploitation are significant for the existence of *G. lucidum.* During stress conditions, *G. lucidum* inevitably remodels its morphological and physiological parameters along with alterations in signaling cascades to modify the metabolic machinery to synthesize bioactive products including secondary metabolites which are the indispensable contributors for acclimation, adaptation and in maintaining homeostasis.

G. lucidum from regions with different environmental and climatic variables such as Punjab (Bathinda), Haryana (Mandi Dabwali) and Himachal Pradesh (Dharamshala and Shahpur) are yet to be explored. Bathinda and its outskirts (Punjab) along with Mandi Dabwali (Haryana) fall within the semi-arid zone, characterized by extreme climatic conditions ranging from low temperature (-2°C) in winter season to extreme high (48°C) in summers, low annual rainfall (20-40 mm), excess of heavy metals (arsenic, mercury, cadmium, selenium, chromium) in soil and water, high pesticides levels in ground water due to unscientific farming practices (Kumar *et al.*, 2011). The

biochemical analysis of *G. lucidum* growing in different fruiting phases (fresh and dry) was performed from the samples collected from these regions and growing on the different host plant. The chemical profiling revealed pharmaceutically active ingredients. The chemical profiling of *G. lucidum* confirmed the presence of myco-constituents with varying amount in response to extreme climatic conditions. *G. lucidum* extracts exhibited an elevated level of total sugars, reducing sugars, starch, proteins, phenols, and antioxidants (DPPH assay) in all myco-constituents but flavonoids, which was more pronounced when in a parasitic relationship with their host plants. The samples collected from Bathinda region with *Azadirachta* and *Acacia* trees as the host plants showed several folds augmentation in levels of *G. lucidum* bio-ingredients as compared to other variants. Elevated levels of myco-constituents observed highlighted their significance in inhibiting proliferation of cancer cells proliferation and reducing ROS in PC-3 cells, thereby confirming that *G. lucidum* growing on host plant *Azadirachta* was more efficient in exhibiting biological activities in comparison to other host plants (Gill *et a*l., 2016).

In order to know about the morphology, chemical composition, and element present in the fruiting bodies of *G. lucidum* under stress condition, FE-SEM analysis was done. Based on this, it was concluded that fruiting bodies in the stress upregulate the level Ca, K, Fe elements which may further help *G. lucidum* to cope with abiotic stress. These elements might bring adaptation with increased level of mycoconstituents in *G. lucidum* in relation with host plant.

GAs were isolated from *G. lucidum* using a well-recognized protocol and its presence was further confirmed by using LC-MS (Keypour *et al.*, 2010). Different peaks in chromatogram ranges within the ranges reported in literature had confirmed the isolation of GAs from *G. lucidum* growing on host plant *Azadirachta*. The mass of different GAs fall in known ranges, which indicates that isolated product is a mixture of GAs, and thus termed as GAs. The isolation of ganoderic acids was followed by analysis of its anticancer potential using colorimetric assay (MTT). Effect of different treatments of ganoderic acids on the growth of cancer cells A549, PC-3, and MDA-MB-231 were assessed in triplicates. Cancer cells were treated with different concentrations (5, 10, 20, 50, and 80 µg/mL) of GAs at different time intervals (24h,

48h, and 72 h). GAs marked 50% of inhibition at 50µg/mL and above concentration and best marked after 48h. The cytotoxic effects of GAs were expressed as IC_{50} values in cancer cells, which meant to find concentration of drug that reduced the absorbance of cancer cells by 50% during treatment. GAs had shown a significant reduction in cell proliferation in all studied cancer cells in a concentration-dependent manner. In A549 cells, decrease in cell metabolic activity at 80 µg/mL concentration was observed after 48h of treatment (50% inhibitory concentration). GAs reduced the metabolic activity (IC_{50}) with 20 µg/mL GAs in PC-3, and MDA-MB-231 cells, respectively. It was interesting to note that inhibition of metabolic activity at 80 µg/mL was more than 65% in PC-3 cells, concluding the fact that the GAs reduced the metabolic activity in PC-3 cells better in comparison to MDA-MB-231 and A549 cells. The cytotoxic effect was analyzed by MTT assay in human peripheral blood mononuclear cells (PBMCs). The GAs didn't show any significant toxicity even at a highest 80µg/mL concentration in PBMCs. Cell viability was checked by trypan blue exclusion test to determine the number of viable or dead cells. GAs inhibited the cell proliferation up to 50% at 50µg/mL concentration in A549, MDA-MB-231, whereas 20 µg/mL in PC-3 cells.

The morphological analysis showed reduction in the density and change in the cellular morphology during treatment with GAs in a dose-dependent manner. GAs treated cells reduced the cell migration to 45%, 52% and 75% in A549, PC-3, and MDA-MB-231 cell, respectively. The reduction in the cell migration is less in 12h and 24h, and it is interesting to note that GAs have more potential to inhibit breast cancer cells as compared to lung and prostate cancer cells.

Intracellular ROS in the cancer cells treated with GAs was carried with H_2DCFDA assay. GAs increased 50% ROS production in cells at 50µg/mL in A549 and PC-3 cells, and at 20µg/mL in MDA-MB-231 cells, respectively, concluding that GAs enhance the rate of production of ROS depending on the cell type. MDA-MB-231 cells produced more ROS when treated with GAs as compared to A549 and PC-3 cells. Despite increases of ROS by H_2DCFDA assay, GAs caused a reduction of ROS in A549, PC-3, MDA-MB-231 cells in NBT reduction assay. The colorimetric data displayed the inhibition of superoxide ion by 50% in A549 cells at 20µg/mL, and

50µg/mL in PC-3 and MDA-MB-231 cells. Thus, it was concluded that GAs exhibit more potential in inhibiting lung cancer than breast and prostate cancer cells in terms of ROS stress.

Colony formation is an important hallmark of metastatic cancer cells to evaluate their reproductive viability. GAs inhibited the colony formation in a dose-dependent manner in cancer cells and reduced colony formation to 27%, 30%, and 50% in A549, PC-3, and MDA-MB-231, respectively. Reduction in the colony formation was more prominent in prostate cancer as compared to breast and lung cancer cells.

Spheroids are capable of forming tumors which generally decreases the response of the drug. GAs caused remarkable reduction in the spheroid aggregation in a dose-dependent manner. GAs cause loosening and distortion of spheroid which caused irregularity in the structure, proving the efficiency of GAs in cancer cells adhesion. Fluorescence microscopy was used to analyze the ROS production by H_2DCFDA assay which exhibited an enhancement in ROS production. GAs caused enhancement in ROS production in MDA-MB-231 cells by 62%, whereas 47%, and 46% in A549 and PC-3 cells, respectively. The generation of ROS may cause the cellular damage resulting in cell death. GAs enhanced the production of intracellular ROS and may nullify the effect of antioxidant enzymes.

GAs also arrest cell cycle in a dose-dependent manner in which cells get arrested in a different phases of three different cell lines. GAs arrested the cell cycle at G0/G1 phase in PC-3 cells. In the case of A549 cells, cells were arrested at G1 phase (47.3%) as compared to the control (29.5%). Interestingly, no alterations in cell cycle were observed in MDA-MB-231 cells. In literature, anti-proliferative effect of *G. lucidumis* reported in HepG2 cells alongwith cell cycle arrest at G1 phase (Liu *et al.*, 2009). Triterpenes present in *G. lucidum* arrest cell cycle at G2/M phase in A549 cells and induce apoptosis by decreasing the expression of Bcl-2 (Feng *et al.*, 2013). The study conducted by Jiang *et al.* reported that *G. lucidum* arrests cell cycle at G2/M phase in PC-3 cells (Jiang *et al.*, 2004). Similarly, alcoholic extract of *G. lucidum* arrests cells cycle at G1 phase in time-dependent manner, in MCF-7 cells (Hu *et al.*, 2002). *G. lucidum* arrests cell cycle at G0/G1 in the MDA-MB- 231 cells, by modulating AP-1 and NF-κB signaling.

Preliminary study indicates the anticancer potential of extracts of *G. lucidum* grown in environmental stress. The main bioactive compound among terpenes is GA which exhibits therapeutics effects. More than hundred isoforms of GAs are known, but few have been explored in field of cancer. Therefore, different isoforms of GAs available in literature were evaluated for exploring mechanistic binding in RTKs in cancer. Alterations in cellular genome results from the progressive accumulation of genetic mutations affecting growth and differentiation which results in cancer progression (Croce, 2008). Chromosomal instability, epigenetic silencing, and changes in the functioning of tumor suppressor genes further advances cancer.

RTKs are transmembrane high-affinity surface receptor regulating numerous functions of cell migration, adhesion, apoptosis, metabolism and cell proliferation (Ullrich *et al.*, 1990). Different studies highlighted the role of RTKs in signaling network involved in various cancers such as prostate, breast, lung and ovarian cancers (Zwick *et al.*, 2001). The multiple molecular mechanisms alter the cellular metabolism by modulating the activity of cell receptors and adapter molecules embedded in the plasma membrane. In normal physiology, cell receptors are embedded in the plasma membranes in inactive form, which on receiving signal get phosphorylated and activated. This, in turn, activates various enzymes, ion channels, and transporters in the downstream signaling pathways (Schlessinger, 2000). Various RTK inhibitors have been designed for active involvement in signal transduction targeting cancer. Previously, it was reported that GA-DM inhibits prostate cancer cell growth and blocks osteoclastogenesis by inhibiting 5a-reductase and androgen receptor (AR) binding activity (Wu, Guo-Sheng *et al.*, 2012). Similarly, ganoderic acid A inhibits JAK-STAT3 signaling pathway in HepG2 cells (Yao *et al.*, 2012). Furthermore, GA-Me down-regulating matrix metalloproteinases 2/9 gene expression and inhibits tumor invasion (Chen *et al.*, 2008). However, these studies were unable to expose the mechanistic binding of GAs, therefore, need to explore binding mechanism was observed. In this study, molecular docking of more than 50 isoforms of GAs was performed on different membrane receptors which initiate and modulate the downstream signaling in various diseases. The receptors were IR, IGFR-1, VEGFR-1, VEGFR-2, and ER. Molecular docking identified the maximum energy,

protein-ligand interactions involved, orientations and conformations best suited for drug preparation depicting the preferred orientation to form a stable complex and the strength of association of binding affinity by different docking parameters.

Additionally, GAs induced the apoptosis after alterations in nucleic acid assessed by PI staining. Cells marked nuclear shrinkage, chromatin condensation, and nuclear fragmentation in GAs treated cells. The previous study of *G. lucidum* (0.25, 0.5, and 1.0 mg/mL) showed nuclear shrinkage, chromatin condensation, and nuclear fragmentation (Jiang *et al.*, 2004). DNA fragmentation is one of the biochemical hallmark of apoptosis, marked by activation of an endogenous endonuclease which cleaves chromatin DNA into inter-nucleosomal fragments. While random fragmentation appears in the process of necrosis, apoptosis-associated DNA fragmentation is characterized by DNA cleavage at regular intervals. The DNA fragmentation does not seem essential for apoptosis but exposes the engagement of chromatin manageable for the disposal by phagocytic cells. Importantly, failures in DNA degradation results in activation and provoking of autoimmune response. In this study, DNA fragmentation was analyzed in control cell and 50 µg/mL GAs treatment cells. GAs induced inter-nucleosomal DNA fragmentation in A549, PC-3, MDA-MB-231 cells, and fragmentation appeared more prominent in treated cells as compared to control cells. The previous study of *G. lucidum* also reported the induction of apoptosis by DNA fragmentation with DNA laddering in PC-3 cells (Jiang *et al.*, 2004). GAs increased the ROS production in dose-dependent manner, which further decreases the mitochondrial membrane potential. The disruption of mitochondrial integrity is an important step in the apoptosis. The process of apoptosis is activated, when a cell starts a cascade of events for its destruction including depolarization of the mitochondrial membrane potential. GAs decreased the mitochondrial membrane potential resulting in permeabilization in the outer mitochondrial membrane and loss of the electrochemical gradient which leads to apoptosis. GAs (50µg/mL) significantly decreased the mitochondrial membrane potential treated for 48h exhibiting 60%, 35%, and 48% decrease in A549, PC-3, and MDA-MB-231 cells, respectively. These results suggest that GAs induced the mitochondrial-mediated apoptosis by disrupting mitochondrial membrane potential. Similar results are reported in a study where MCF-

7 cells after 24h treatment with GA-DM induced mitochondrial membrane depolarization, in a concentration-dependent manner (Wu, Guo Sheng *et al.*, 2012). Similarly, GA-Mf or GA-S cause decrease of mitochondrial membrane potential in dose-dependent manner in HeLa cells (Liu *et al.*, 2011).

The process of apoptosis is regulated and controlled by different signaling pathways, like PI3K/Akt/mTOR contributing in growth and proliferation of cells. GAs down-regulated the expression level of PI3K, Akt, and mTOR in dose dependent manner. GAs decreased the expression level of PI3K to 46% at 50µg/mL in A549, 32% in PC-3 and 53% in MDA-MB-231 cells. Akt expression was suppressed to 34% in A549, 56% in PC-3, and 49% in MDA-MB-231 cells. Furthermore, GAs decreased the expression level of mTOR to 54% in A549, 39% in PC-3, and 48% in MDA-MB-231 cells. Thus, it can be concluded that GAs decrease the expression of PI3K, Akt, and mTOR many-folds and target signaling effectively. Baselga in his study reported that *G. tsugae* phosphorylates the human epidermal growth factor receptor 2 (HER2) and activates the PI3K/Akt and Ras/mitogen-activated protein kinase (MAPK) in cancer (Baselga *et al.*, 2009). The ethanolic extract of spores of *G. lucidum* (SBGS) arrests cell cycle at G2/M phase, and decreases the expression of cyclin B1, cdc2, Bcl-2 and Bcl-xl in lung cancer (Chen *et al.*, 2016). Thus, it can be concluded that difference in suppression may be due to different nature and origin of cells, their specification and responses to various receptors.

Similarly, expression of MMP-2 and MMP-9 was analyzed and marked decrease of protein expression was observed in A549, PC-3, and MDA-MB-231 cells. GAs significantly inhibited MMP-2 expression in lung cancer cells by one fold, prostate cancer by three-fold, and breast cancer cells by two fold. On the other hand, MMP-9 expression decreased in lung cancer by one fold while two folds in prostate cancer and breast cancer cells. From above results, it can be concluded that GAs target MMPs better in prostate cancer than lung and breast cancer cells. GAs decreased the MMPs expression and induced apoptosis. Studies conducted previously showed that GA-Me down-regulated the expression of MMP 2/9, whereas GA-Mf and GA-S induce apoptosis and arrest cell cycle in 95-D cells (Liu *et al.*, 2011). Apoptosis is controlled and maintained by balance of bax/bcl-2 ratio deciding the fate of the cell. In

this study A549 cells, GAs up-regulated the expression of bax by one fold, and decreased the Bcl-2 expression by one fold. GAs increased the expression of bax by two fold, and decreased the expression of Bcl-2 by one fold in PC-3 cells. In MDA-MB-231 cells, GAs up-regulated bax expression by one fold and but didn't causes any change in Bcl-2 expression. Thus, it can be concluded that change in bax/bcl-2 ratio results in induction in the process of apoptosis. *G. lucidum* (0.25, 0.5, and 1.0 mg/mL) in the previous studies, shows the decreased in Bcl-2 and bcl-xl expression with increase in Bax expression in PC-3 cells in a time-dependent manner (Jiang *et al.*, 2004).

In this study, GAs modulated the expression of apoptosis associated proteins, as suggested by decrease in bax and increase in Bcl-2 expression. These changes in the expression, alter the crucial balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins, which prompts the loss of mitochondrial membrane potential, responsible for mitochondria-mediated apoptosis. GAs effectively inhibited the cell growth, migration, colony formation, spheroid formation in a dose-dependent manner. GAs induced the apoptosis through a nuclear breakdown, DNA fragmentation, morphological changes in cancer cells controlling the cell cycle in the apoptosis process. GAs induced the process of apoptosis by altering the expression of Bcl-2 and bax proteins. Bcl-2 family proteins are the regulators of apoptosis and balance between the ratio of pro-apoptotic and anti-apoptotic gene expression decides the nature of apoptosis. In this study, GAs target apoptosis in cancer cells, but different isoforms present in nature make it uncertain about the effect of particular isoform in targeting apoptosis. Therefore, further research needs to be done to isolate particular isoform targeting a specific protein in apoptosis. In order to have look, the mechanistic binding of available isoforms (more than 50) was targeted computationally exposing the binding interaction among various proteins.