2.0. Cellular stress and diseases

It is widely known that protein specifically folds to form three dimensional structures to be active and functional but during unfavorable conditions such as environmental, physiological and pathological disturbances, the synthesized proteins undergo aberrant folding and aggregation, potentially forming toxic species. Cellular stress refers to any stimulus that disrupts the protein homeostasis and function. The stress proteins or molecular chaperones are the important players in cytoprotection against various cellular insults. Molecular chaperones are the large protein molecular machinery that has the remarkable ability to assist folding of proteins to their native state using ATP inside the cell and thereby aiding in cell survival [Hartl et al., 2011].

The process of robust and rapid production of stress proteins in organisms upon exposure to protein damaging cellular stresses is known as “heat shock response. For the first time, Ritossa [1962] discovered heat shock response in chromosomal puffings on salivary gland chromosomes of Drosophila buscii. Subsequently, it was perceived that heat shock treatment induced synthesis of novel class of proteins that were similar in different tissues of Drosophila melanogaster, while the levels of other proteins were reduced [Tissieres et al., 1974].

Mutations in the genes encoding several molecular chaperones result in neurological, muscular, or cardiac age-related diseases in humans. The malfunction of molecular chaperones has been implicated in abnormal placental development and preterm deliveries, heart failure, atherosclerosis, aging, fever, infection, malignant diseases, alzheimer’s disease and, autoimmune disorders. The cyto-protective actions of molecular chaperones have an adverse effect directed to favor tumor growth and metastasis among breast cancer, leukemia, pancreatic and ovarian cancer [Whitley et al., 1999].
2.1. Heat shock proteins and inhibitors

Heat shock proteins (HSPs) are subsets of molecular chaperones expressed to maintain cellular homeostasis under normal conditions. HSPs are essentially housekeeping proteins that are involved in assembly or disassembly of other macromolecules participating in metabolism, assisting in repair and refolding of misfolded proteins to avoid their aggregation and also assist in nascent protein folding. These proteins are ubiquitous, present in all kingdoms of life. Most of the HSPs have conserved sequences ranging from bacteria to human [Solárová et al., 2015].

Cells respond to unfavorable environmental stimulus (cold, heat, temperature fluctuation, change in pH, hypoxia etc.) and diseases like cancer by increased synthesis of stress proteins such as HSPs. There are four major subclasses of HSPs: HSP90, HSP70, HSP60, and small HSPs (<40 kDa). Among these, overexpression of HSP90 and HSP70 are observed in both solid tumors and hematological malignancies and play a crucial role in maintaining oncogenic protein homeostasis [Tutar, 2015]. Due to accumulation of misfolded proteins, the proteotoxic stress in cancer cells compels it to rely upon HSP70 and HSP90 that stabilizes, maintains and regulates oncogenic client proteins such as HER-2, Cyclin-dependent kinase 4 (Cdk-4), Protein kinase B (Akt/PKB), Rapidly accelerated fibrosarcoma (Raf-1) for survival and proliferation. The overexpression of HSPs in cancer cells correlates to either poor prognosis or accelerated resistance to anticancer therapies, thus allowing the cell to get through lethal conditions and mediates tumorigenesis [Stope et al., 2016; Tutar, 2015]. Therefore, inhibition of HSPs has been recognized as potential therapeutic strategy for cancer treatment for the last two decades.

HSP90 is one of the most abundant molecular chaperones present in unstressed eukaryotic cells that accounts for 1-2% of cellular proteins. Its expression was found to be
2-10 folds higher in malignant cells compared to normal cells. HSP90 family comprises both inducible and constitutively expressed cytosolic isoforms namely HSP90α and HSP90β. Furthermore, HSP90 has GRP-94 and TRAP1 isoforms in the endoplasmic reticulum and mitochondria respectively [Li and Buchner, 2013].

**Figure. 2.1.** (A) Crystal structure of HSP90. (B) Conserved domains of HSP90. There are three functional domains in HSP90: N-terminal domain (NTD), middle domain (MD) and C-terminal homodimerization region (CTD).

Briefly, the structure of HSP90 is a homodimeric protein that comprises three conserved functional domains: N-terminal domain (25 kDa), middle domain (55 kDa) and C-terminal domain (10 kDa). The N-terminal domain (NTD) has an ATP binding site, involved in hydrolysis of ATP to ADP facilitating large conformational changes and interaction with client proteins and co-chaperones HSP40 and HSP70. The C-terminal domain ends with a MEEVD-peptide motif which is recognized by co-chaperones carrying a tetratricopeptide repeat (TPR) domain such as HSP organizing protein (HOP), FK506-binding protein (FKBP52) and protein phosphatase 5 (PP5). The middle domain possesses large hydrophobic surface that permits binding of cochaperones and client proteins (Fig. 2.1).
Chapter 2: Review of literature

The HSP90 complex cycles from the ADP-bound state to the ATP-bound state and the conformational change that occurs with replacement of ADP by ATP stabilizes and activates client proteins. When ADP is bound to HSP90, the client protein is released and may be marked for destruction by the proteasome through ubiquitination (Fig. 2.2). To date, over 175 client proteins are involved in a multitude of cellular activities such as cell cycle control; proliferative/antiapoptotic signaling and many are activated in malignancy [Richardson et al., 2011]. Oncogenic client proteins are over expressed in all types of cancers and play an important role in the regulation of the cell cycle, cell growth, cell survival, anti-apoptosis, angiogenesis, and oncogenesis.

Figure 2.2. Heat shock protein 90 (HSP90) in vivo. The client protein binds to the co-chaperones HSP70 and HSP40 and then is loaded onto the HSP90 homodimer. Subsequent binding of adenosine triphosphate (ATP) causes a conformational change that stabilizes the client proteins for interactions with other ligands or stimuli. If no further interaction occurs, hydrolysis of ATP to adenosine diphosphate (ADP) facilitates the release of the client protein, which can undergo degradation through the proteasome. The dissociation of ADP from the HSP90 homodimer restores HSP90 to its open conformation [Den and Lu, 2012].

HSP90 plays a key role in stabilizing various oncogenic client proteins such as p53, v-Src, Akt, Her-2, MET, EGFR, ErbB2, HIF1-α, Bcr-Abl, Cdk6, c-met, survivin and telomerase [Ozgur and Tutar, 2014]. Thus, HSP90 is able to protect client proteins from
misfolding in the presence of cellular stress. HSP90 participates in post-translational modification and stabilization of a number of conformationally labile proteins, steroid receptors, cyclin-dependent kinase 4, Raf-1, AKT and other proteins that are useful for sending proliferative signals [Whitesell et al., 1994]. Therefore, once the function of HSP90 is blocked, its client proteins are eventually degraded by proteasomes.

Figure. 2.3. Chemical structures of natural products of HSP90 inhibitors.

Owing to the implication of HSPs in cancer, preclinical phase studies and trials emphasize the importance of HSP90 and HSP70 inhibitors. HSP90 inhibitors are ATPase activity inhibitors (Fig. 2.3) that aims to deregulate the HSP90 folding. These inhibitors may be categorized into two main classes: natural inhibitors (geldanamycin, 17-AAG, 17-DMAG, herbimycin, radicicol, novobiocin, epigallocatechin 3-gallate, IPI-504, derrubone, gedunin, celastrol and their derivatives) and synthetic inhibitors (purine scaffold inhibitors, pyrazole scaffold inhibitors, SNX-2112, STA9090 and their derivatives) [Li et al., 2009]. These agents were subsequently shown to bind to HSP90 and structural studies have demonstrated that they compete with ATP for binding at the catalytic site of the N-
Chapter 2: Review of literature

terminal domain. This results in inhibition of the essential ATPase activity that drives the molecular engine of the chaperone. Of these, geldanamycin is the first natural HSP90 inhibitor which was isolated from *Streptomyces hygroscopicus*. Later, synthetic geldanamycin derivatives were synthesized. Additionally, geldanamicyn and 17-AAG have shown clinical benefit in Her2-positive metastatic breast cancer [Modi, 2009] but are limited by hepatotoxicity, bioavailability, extensive metabolism, poor solubility and the need for solvent carrying agents [Workman, 2004]. Another natural product, radicicol, is also a potent inhibitor of HSP90 ATPase activity [Roe et al., 1999] but the molecule has a number of unfavorable pharmacological properties and *in vivo* instability [Soga et al., 1999]. The PU-H71 another HSP blocker has shown complete response in TNBC models [Caldas-Lopes et al., 2009].

One of the major side effects of HSP90 inhibitors is the compensatory induction of HSP70 expression – a potent negative regulator of cell death. HSP70 synthesis is mainly regulated by transcriptional factor heat-shock factor 1 (HSF1) [Ciocca et al., 2013].

Hence isolation and validation of plant adaptogens may lead to greater efficacy and improved clinical outcomes. In the present scenario, alternative inhibitors from plant origin targeting HSP90 and HSP70 are of utmost importance. Curcumin (*Curcuma longa*), a medicinal herbal compound is capable of inducing the HSP70 [Dunsmore et al., 2001]. Flavonoids (*Atractylodes lancea*) inhibited the expression of HSP27, HSP47, HSP60 and HSP72/73 [Morino et al., 1996]. Withaferin A, a steroidal lactone occurring in *Withania somnifera* inhibits HSP90 chaperone activity resulting in HSP90 client protein degradation, and exhibits *in vivo* anticancer activity against pancreatic cancer [Yu et al., 2010]. Compounds like shikonin from *Arnebia hispidissima* showed significant HSP70 up
Chapter 2: Review of literature

regulation [Ahmed et al., 2012]. Hence, most drugs that target HSPs, which is more beneficial than the selective oncogene pathway inhibitors.

The consequence of upregulation of anti-apoptotic and cytoprotective HSP70 protein is believed to reduce the overall anti-tumor efficacy of many compounds [Leu, 2011]. Therefore, exploring simultaneous inhibitions of HSP90 and HSP70 could be a promising therapeutic approach and may suggest a future direction for drug development of HSP inhibitors. Combinatorial therapies seem to be an effective way to target various cancers, applying low doses of these drugs together with conventional chemotherapeutics.

2.2. HSF-1

Besides maintaining cellular homeostasis in normal cells, HSPs play a crucial role in the proper folding of large number of client proteins involved in promoting cancer cell growth and/or survival and also aids in the interaction between many receptor tyrosine kinases (RTKs) and their substrates, which presumably are involved in tumor angiogenesis and loss of apoptosis [Mancini et al., 2014; Rao et al., 2012]. HSPs are essentially over expressed in many cancers; specifically HSP90 and HSP70 are reportedly over expressed in breast cancer cells [Chiosis et al., 2013; Du et al., 2014; Zagouri et al., 2010]. Their expression is in turn dependent on the activation of Heat shock factor-1 (HSF-1), a master regulator and a transcription factor which has recently been shown to drive neoplastic transformation by modulating diverse pathways that regulate survival, proliferation, protein synthesis, and cellular metabolism [De Thonel et al., 2011]. HSF-1 is constitutively activated in tumors and often correlates to poor prognosis of breast cancer patients.

Normally, HSF1 shuttles between the cytoplasm and the nucleus, but upon activation by proteotoxic stress as in cancer, it concentrates in the nucleus [Dai et al., 2012]. Under unstressed state, HSF-1 is present in cytoplasm as an inactive monomer bound by
HSP90, HSP70. But during proteotoxic stress in cancerous cells, HSF1 is released from the chaperone complex [Westerheide et al., 2006].

The activation of human HSF1 is a two-step process beginning with an initial step of homotrimer formation, that are able to bind to HSE (heat shock element) sequences present on hsp genes and in the second step, these HSF1 homotrimers gain transcriptional activity by phosphorylation at specific residues (Fig. 2.4).

**Figure 2.4. The heat shock response pathway.** Accumulation of unfolded proteins initiates the release and subsequent trimerization and hyper phosphorylation of HSF1. HSF1 then binds DNA and initiates upregulation of HSP mRNA and protein, including HSP90 and HSP70 [McConnell et al., 2015].

Phosphorylation of human HSF1 on Ser\textsuperscript{230}, Ser\textsuperscript{320}, Ser\textsuperscript{326} and Thr\textsuperscript{142} residues by Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA), mammalian target of rapamycin complex 1 (mTORC1) and Casein kinase 2 (CK2)
respectively is known to activate it by augmenting its transcriptional capability [Santagata et al., 2011b]. Furthermore, human HSF1 has also been shown to be repressed as a result of phosphorylation on Ser\textsuperscript{121}, Ser\textsuperscript{303}, Ser\textsuperscript{307} and Ser\textsuperscript{363} residues by MK2 kinase, GSK3\textbeta, ERK and JNK/SAPK or protein kinase C (PKC) respectively. Apart from regulation of expression of HSPs, HSF1 has been shown to regulate tumor angiogenesis and metastasis. Knockdown of HSF1 strongly suppresses accumulation of HIF1\alpha and also VEGF both \textit{in vitro}, \textit{in vivo} [Gabai et al., 2012] and also overexpression of HSF1 significantly correlated with tumor angiogenesis in patients with non-small cell lung cancer (NSCLC) [Cui et al., 2015]. Likewise, HSF-1 has also been identified as one among the six potent metastasis promoting genes [Scott et al., 2011] and is associated with potentiating metastasis in hepatocellular [Fang et al., 2012], prostrate [Hoang et al., 2000; Tang et al., 2005], breast, colon and lung cancers [Mendillo et al., 2012].

Compounds such as triptolide, quercetin, benzylidene lactam and emunin have been reported as HSF1 inhibitors [Whitesell and Lindquist, 2009]. Also, a thiazole nucleoside analog, HIV protease inhibitor Ritonavir, sulphoraphane and phenethylisothiocyanate suppresses HSF1 expression in pancreatic cancer cells [Xia et al., 2012], renal cancer cells [Sato et al., 2012] and breast cancer cells [Sarkar et al., 2012].

High-throughput screening for new HSF1 inhibitors have yielded promising small molecules (limonoids, curcularins, withanolides, celastraloids, and colletofragarones) with potent HSF-1 inhibiting capability, but are not specific HSF1 inhibitors [Santagata et al., 2013; Santagata et al., 2011b]. However, KRIIB11, a cell-permeable 2, 6-diaminopyridine compound is a more authentic HSF1 inhibitor. Its interaction with HSF1 is reversible and blocks the transcription of HSPs and is reported to have antiproliferative activity in cancer cell lines and regressed HCT116 tumor growth in mice [Yoon et al., 2011].
2.3. Tumor angiogenesis

Tumor angiogenesis is one of the best targets for cancer therapy. Tumor cells do not shed into the circulation in the absence of angiogenesis. The tumor cells being genetically unstable, causes progressive increase in the number of different angiogenic factors that helps cancer progression to further advanced stages. The complex pathways of angiogenic system provide many promising therapeutic targets. Also, the redundancy in the angiogenic pathways raises the possibility of resistance to selective therapeutic agents. Examples of agents that target circulating angiogenic factors include monoclonal antibodies (bevacizumab) targeted against VEGF [Willett et al., 2004] or fusion proteins (afiblercept or AMG386) that trap angiogenic factors [Lockhart et al., 2009]. Inhibitors of HSP90, mTOR or cyclo-oxygenase (COX) have also been shown to antiangiogenic in nature [Zhong et al., 2000; Miyata et al., 2013]. These agents in combination with antiangiogenic factors can inhibit several other aspects of cancer biology such as growth, resistance to apoptosis or metastasis.

Axitinib, pazopanib, regorafenib, sunitinib and sorafenib are the examples of tyrosine kinase inhibitors used to target angiogenic signaling in lung, colorectal, breast, gastric, glioblastoma, hepatocellular and neuroendocrine tumors [Gotink and Verheul, 2010; Mihaly et al., 2010; Grothey et al., 2013]. Small molecules that competitively bind to receptors involved in angiogenesis signaling are currently been used in the treatment of numerous malignant tumors aimed at blocking lymph angiogenesis/ metastasis via neutralizing VEGF-A, -C or -D-induced receptor activation. For example, a major approach involves application of a variety of tyrosine kinase inhibitors such as Ki23057, used to block the spread of gastric cancer in mice through blockade of VEGFR3 autophosphorylation [Yashiro et al., 2009]. Cyclophosphamide, methotrexate and
capecitabine are the examples of metronomic chemotherapeutic drugs that have antiangiogenic activity [Penel et al., 2012; Kerbel, 2011].

Paclitaxel, doxorubicin and thalidomide are the other chemotherapeutic drugs that exhibit anti-angiogenic activity via VEGF and bFGF inhibition both in vitro and in vivo models [McMeekin et al., 2007]. Celecoxib, which may cause a time-dependent reduction in circulating angiogenic markers [Brugnoli et al., 2014]; bisphosphonates may have anti-angiogenic effects via reduction of VEGF and PDGF serum levels [Santini et al., 2003]. Analogues of Rapamycin such as temsirolimus and everolimus decrease tumor angiogenesis via inhibiting mTOR activity [Battelli and Cho, 2011].

2.4. Implication of HSPs in tumor angiogenesis

To grow beyond the diffusion distance of oxygen in tissue (100 mm), tumors must assemble an ad hoc microcirculation through de novo angiogenesis [Folkman, 2002]. HSPs are important in this process through their influence on the primary sensor of tumor cell hypoxia – the transcription factor HIF-1α [Zhou et al., 2004]. This factor is regulated at the level of protein stability, and increased amounts of both HSP70 and HSP90 are needed to mediate its stabilization and accumulation.

A variety of HSP90 inhibitors have been developed in the past decade and have shown convincing anti-neoplastic activity in pre-clinical tumor models. HSP90 inhibitors are predominantly being recognized to target tumor cells. Cancer cells overexpress HSP90 protein, thereby depends on HSP90 function for maintaining oncogenic signaling. HSP90 inhibitors bind with high affinity to HSP90 in tumor cells. Nevertheless, results from recent studies also suggest that HSP90 inhibitors elicit anti-angiogenic properties by affecting the PI-3K/Akt/eNOS signal transduction pathway in endothelial cells, as well as through down-regulation of VEGFR-2 expression, a classical component of the angiogenic process.
[Calderwood et al., 2006]. In addition, blocking HSP90 may also diminish the secretion and expression of tumor cell-derived pro-angiogenic growth factors and cytokines, thus leading to “indirect” anti-angiogenic effects [Staufer and Stoeltzing, 2010].

2.5. Apoptosis

Apoptosis is the programmed cell death which maintains the healthy survival or death balance in metazoan cells. Defective proteins involved in apoptosis can cause cancer or autoimmunity, while enhanced apoptosis may cause degenerative diseases. Accordingly, the apoptotic signals contribute into safeguarding the genomic integrity while defective apoptosis may promote carcinogenesis [Hassan et al., 2014]

There are two major pathways by which apoptotic cell death can be induced: The intrinsic (or mitochondrial) pathway and the extrinsic (or death receptor) pathway. The intrinsic pathway regulates the activity of proteins of the survivin and B-cell lymphoma 2 (BCL-2) families. The latter family includes myeloid cell leukemia-1 (MCL-1), which plays an integral role in cell survival and apoptosis [Sano et al., 2008], BCL-2-associated X protein (BAX), a pro-apoptotic protein that induces the release of cytochrome c from mitochondria to the cytosol where it binds to apoptotic peptidase activating factor 1 and facilitates the formation of the apoptosome, leading to the activation of caspase-9 and eventual cell death. The extrinsic pathway is activated by specific ligands that engage death receptors. This process involves Fas, which binds to and activates the caspase-8 protein [Arnoult et al, 2002; Fulda and Debatin, 2006]. The tumor cells may use some of the several molecular mechanisms to suppress apoptosis and acquire resistance to apoptotic agents, for example, by the expression of antiapoptotic proteins such as BCL-2 and proapoptotic proteins such as BAX [Hassan, 2014].
Anticancer drugs could induce apoptosis by production of ROS in a variety of tumor cells via the activation of caspases and disruption of mitochondrial membrane potential. Mitochondria are the major site of ROS production, and accumulation of ROS may lead to the initiation of apoptosis. The cell death induction is associated with Bax oligomerisation, release of cytochrome c, caspase activation, and internucleosomal degradation [Liou and Storz, 2010].

During apoptosis, several events such as chromatin condensation and fragmentation of the nucleus and cytoplasm, protein cleavage, DNA breakdown and recognition of the dying cell by phagocytes result in distinctive structural modifications [Hengartner, 2000]. The main inhibitors of these changes are a family of cysteine proteases called caspases. Caspases are expressed in cells in inactive proenzyme form, which once activated through proteolysis, can activate other procaspases leading to the initiation of a protease cascade. In this proteolytic cascade, caspases cleave a host of cellular substrates, leading to the morphological hallmarks of apoptosis, including DNA fragmentation. Caspase-8 or -9 acts as an initiator of apoptosis, whereas others (e.g., caspases-3 and -7) serve as effectors [Salvesen and Dixit, 1997]. Thus, caspases are central regulators of the apoptotic process, and are involved in the two major apoptosis pathways [Debatin, 2004].

2.5.1. Akt and p53

The Akt/ PKB kinase and the p53 tumor suppressor protein have a significant role in the apoptotic signals. Akt/PKB is an antiapoptotic protein while the p53 is a pro-apoptotic protein [Gottlieb et al., 2002]. In the breast cancer cell lines, HSF-1 is directly phosphorylated by Akt and regulates its stability, transcriptional activity and intracellular trafficking. Akt is usually dysregulated and hyperactive in human tumors and thus becomes an important target for cancer therapeutics [Gabai et al., 2012].
Chapter 2: Review of literature

In unstressed cells, p53 is present in an inert state and is maintained at low levels. Various genotoxic stresses such as oncogene activation, reactive oxygen species (ROS) or nutrient starvation, initiate signaling pathways that stabilizes the p53 protein, cause it to accumulate in the nucleus, and activate it as a transcription factor. The activation further leads either to growth arrest at the G1/S or G2/M transitions of the cell cycle or to apoptosis [Appella and Anderson, 2001]. p53 is phosphorylated at multiple sites in vivo and by several different protein kinases in vitro. DNA damage induces phosphorylation of p53 at Ser15 and leads to a reduced interaction between p53 and its negative regulator, the oncoprotein MDM2. MDM2 along with MDM4 inhibits p53 accumulation by targeting it for ubiquitination and proteasomal degradation. Also, the phosphorylation in the p53 at ser15 protein can trigger many subsequent functionally important post translational modifications of the p53 protein [Loughery et al., 2014].

On the other hand, in the presence of appropriate survival signals, Akt activation by phosphorylation within the carboxy terminus at Ser473 may lead to MDM2 phosphorylation [Sarbassov et al., 2005]. This is speculated to potentiate MDM2, and thereby incapacitate p53 and overcome its pro-apoptotic effects [Gottlieb et al., 2002].

2.6. Implication of HSPs in Apoptosis

The overexpression of HSP90 and 70 is known to block apoptosis. These proteins promote drug resistance in different forms of cancers like oral, breast, prostate, endometrial, colorectal cancers as well as leukemia mediated by HSF1 [Murphy, 2013]. HSP70 regulates key mediators of apoptosis by blocking expression of TNF, activation of caspase-3, translocation of BAX and cleavage of Peroxisome proliferator-activated receptor (PPAR) [Evans et al., 2011]. HSP70 over-expression allows resistance to chemotherapeutic agents, like MG-132, imatinib, etopiside and cisplatin. Recent evidence suggests that
Chapter 2: Review of literature

resistance is because of reduced activation of ERK, NF-κB, and JNK pathways [Gabai, 2005; Bagatell, 2000].

The anti-apoptotic action of HSP90 is also mediated by its interaction with phosphorylated serine/threonine kinase Akt/PKB, a protein that generates a survival signal in response to growth factor stimulation. Binding of HSP90 protects Akt from protein phosphatase 2A (PP2A)-mediated dephosphorylation and inactivation [Sato et al., 2000]. Phosphorylated Akt phosphorylates the BCL-2-related pro-apoptotic protein BAD and caspase-9, thereby inhibiting the intrinsic pathway to apoptosis [Cardone et al., 1998]. In addition, active Akt phosphorylates I-κB kinase, resulting in promotion of NF-κB-mediated inhibition of apoptosis [Parcellier et al., 2003].

Like HSP70, HSP90 is an ATP-utilizing molecular chaperone with roles in protein turnover. However, HSP90 is often considered the more “specialized” chaperone, with a relatively restricted set of cellular substrates. Inhibition of binding of HSP90 to ATP leads to proteasomal degradation of these substrates and corresponding anti-proliferative activity. In the context of our discussion, treatment with HSP90 inhibitors has also been found to induce expression of HSP70, likely through activation of HSF1. As discussed above, this compensatory mechanism can cause resistance to apoptosis and stabilization of some shared protein substrates, such as Akt [Evans et al., 2010].

2.7. Reactive oxygen species in cancer

Many pathological and physiological events like xenobiotics exposure, diseases like cancer, neurodegenerative diseases, and arteriosclerosis causes oxidative stress by producing ROS. High amount of ROS is generated in cancer cells compared to normal cells making the cell more susceptible to the additional ROS assault from the exogenous agents. The unrestricted accumulation of ROS leads to apoptosis, necrosis and autophagy.
Apoptosis is an active cellular death process induced by normal physiological or pathological factors for the elimination of unwanted or damaged cells. There are reports suggesting that ROS mediated apoptosis is induced via activation of p38, MAPK and JNK [Zhang et al., 2015].

The mechanisms of apoptosis are very complex and regulated in an orderly way involving cascades of energy-requiring molecular events. Plant derived compounds have been shown to induce apoptosis in MDA-MB-231 cells by intrinsic as well as extrinsic pathways. Carnosol is a naturally occurring polyphenol that triggers the activation of both the intrinsic and extrinsic apoptotic pathways in MDA-MB-231 cells [Al Dhaheri et al., 2014]. Resveratrol, a natural polyphenol found in grapes coulds significantly increased the apoptosis in MDA-MB-321 cells [Garvin et al., 2006]. Ampelopsin, a phytochemical oligostilbenoid constituent isolated from Dryobalanops caused G2/M phase cell cycle arrest and induced apoptosis in triple negative breast cancer cells, MDA-MB-231 [Rahman et al., 2016]. Piperine is another alkaloid isolated from Piper nigrum and Piper longum that exhibits TRAIL based cytotoxicity in TNBC cells [Abdelhamed, 2014]. Treatment of anacardic acid purified from the leaves of Anacardium occidentale, induced apoptosis in TNBC cells by the regulation of p53, MAPK and NF-κB pathways [Raj et al., 2016].

There are several studies which indicate the inhibition of MDA-MB-231 proliferation from natural products, treatments with withaferin-A [Hahm et al., 2011], deltonin [Zhang et al., 2013] vernodalin [Looi et al., 2013], combination of curcumin and arabinogalactan [Moghtaderi et al., 2017] induce apoptosis mediated by mitochondria-derived ROS production in MDA-MB-231. This is coupled with downregulation of anti-apoptotic molecules like BCL-2, BCL-xL and release of cytochrome c. Release of cytochrome c from
mitochondria to cytosol triggers the activation of caspase cascade, PARP cleavage, DNA damage and eventually cell death.

### 2.8. Compounds from plant source for cancer inhibition

During the last decade, phytochemicals have gained significant recognition for their potential therapeutic uses in cancer treatment and extensive research has revealed enormous potential pharmacological properties of plant-based medicinal compounds, and demonstrated synergistic effects in combination with other agents to inhibit cancer. Some phytochemicals used in cancer therapies demonstrate relatively low side effects, and some even limit the side effects of anti-cancer drugs [Atanasov, 2015].

The phytochemicals from many medicinal herbs, fruits, vegetables, cereals, pulses, legumes, herbs, spices have anti-angiogenic and pro-apoptotic properties in different cancers targeting different molecules, receptors, proteins via different signaling pathways. Some of the examples are Curcuma longa (curcumin, genistein), Vitis vinifera (quercetin), Solanum nigrum (solamargine and solasonine), Camellia sinensis (epigallocatechin gallate), Withania somnifera (withanolides- withaferin A, sitoindoside IX, physagulin D, withanoside IV, viscosalactone B), Ocimum sanctum (linolenic acid, rosmarinic acid), Zingiber officinalis (6-gingerol, 6-paradol, shogaols and zingerone), Emblica officinalis (proanthocyanidins, ellagic acid, gallic acid, quercetin, kaempferol, emblicanin), Catharanthus roseus (vinca alkaloids such as vinblastine, Vincristine), Ginkgo biloba (ginkgetin and ginkgolides), Glycine max (Isoflavones such as genistein and daidzein), Glycyrrhiza glabra (glycyrrhizin), Gossypium hirsutum (gossypol), Prunella vulgaris (ursolic acid, oleanolic acid), Saussurea lappa (cynaropicrin, costunolide), Viscum album (viscumin, digallic acid), Citrus limon (limonoid, limonene), Daucus carota (polyacetylenes), Vitis vinifera (resveratrol). There are many such plants considered to be...
good sources of phytochemicals having anti-angiogenic and pro-apoptotic activities in various cancers. The active compounds in these plants are sometimes extracted and given in doses higher than what can be achieved from consuming the plants of which they are derived in order to give stronger therapeutic effect [Umadevi et al., 2013; Pandey and Madhuri, 2009; Zaini, 2012; Nivelle, 2017]. N-methylhemeanthidine chloride (NMHC) is a novel alkaloid isolated from the plant Zephyranthes candida (Amaryllidaceae) inhibits acute myeloid leukemia through inhibiting NOTCH signaling pathway, for which commercial drugs are in development. There are also other phytochemicals, which exhibit such effects [Ye, 2016].

*Ganoderma lucidum*, a popular medicinal mushroom suppresses angiogenesis by regulating MAPK and Akt signaling, inhibiting AP-1 (activator protein) activation, and the down-regulation of VEGF, transforming growth factor (TGF)-beta [Stanley et al., 2005], MMP-9, as well as the upregulation of TIMP-1 which lead to reduction of tumor cell invasion and blood vessel growth [Liu et al., 2010].

### 2.8.1. Compounds from plant source for breast cancer inhibition

With successful clinical trials, drugs being developed from plant origins are popular for clinical development. Their non-toxic effects on normal cells and their cytotoxic effects on cancer cells put them in high demand. There are many natural products isolated from plant source which exert chemopreventive activity against breast cancer, such as denbinobin, curcumin, ursolic acid, genipin, sauchinone, lycopene and capsaicin.

Curcumin [1, 7- bis (4-hydroxy-3-methoxyphenyl) 1, 6-hepta-diene- 3,5-dione] is a natural dietary pigment isolated from the root of the plant *Curcuma Longa Linn* (turmeric) triggers apoptosis in breast cancer through the PI3K/Akt signaling pathway [Lv et al., 2014; Kizhakkayil et al., 2010]. Ursolic acid (UA) is a pentacyclic triterpenoid compound
extracted naturally from herbs. CyclinD1/CDK4 correlates with cell cycle progression and cancer progression [Lamb et al., 2013] and FoxM1, a transcription factor, is a key for cell proliferation and cell cycle progression. UA-induced apoptosis decreases cyclinD1/CDK4 expression through regulation of FoxM1 in MCF-7 human breast cancer cells [Wang et al., 2012]. Genipin, an active molecule of the plant Gardenia jasminoides Ellis, induces apoptosis and inhibits invasion in MDA-MB-231 breast cancer cells by downregulating BCL-2, upregulating BAX and caspase-3, as well as the pro-apoptosis products JNK and p38 MAPK [Kim et al., 2012]. Mitochondrial uncoupling protein (UCP2), which promotes tumorigenic properties, is over-expressed in MCF7 human breast cancer cells. UCP2 is also associated with cell viability through regulation of ROS production, apoptosis, and autophagy. Inhibition of UCP2 involves an increase in ROS production, apoptosis, autophagy and a decrease in cell viability. Thus, genipin decreases cancer cell viability by inhibiting UCP2 [Pons et al, 2015]. Sauchinone, a bioactive constituent extracted from the root of Saururus chinensis could induce apoptosis in MCF-7 cells through control of BCL-2, caspase-3, VEGF, cyclin D1 and the extracellular signal-regulated kinase (ERK) signaling pathway [Kim et al, 2011]. Lycopene is the major carotenoid in tomatoes that inhibits proliferation of H-Ras-transformed MCF10A human breast cells and MDA-MB-231 human breast cancer cells by activating ERKs and inhibiting Akt [Koh et al, 2010]. Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is a natural pungent ingredient found in red pepper that has showed anticarcinogenic activity in MCF-7 breast cancer stem cells by inducing apoptosis through Notch signaling [Shim and Song, 2015]. These compounds not only have the capacity to trigger cell death but also synergize apoptosis triggered by numerous anticancer drugs in several tumor cell lines by interfering with multiple pathways leading to chemo resistance [Ko and Moon, 2015].
### Table 1: Effects of natural products for chemoprevention of breast cancer [Ko and Moon et al., 2015]

<table>
<thead>
<tr>
<th>Natural product</th>
<th>Mechanism</th>
<th>Related factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsaicin</td>
<td>Apoptosis</td>
<td>ROS, Rac1, c-Jun, JNK-1, p38, caspase-3</td>
</tr>
<tr>
<td></td>
<td>Cell cycle transition</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-proliferation</td>
<td>EGFR/HER2 signaling pathway</td>
</tr>
<tr>
<td>Chalcone</td>
<td>Apoptosis, anti-angiogenesis</td>
<td>VEGF/VEGFR-2 signaling pathway</td>
</tr>
<tr>
<td>Codonolactone</td>
<td>Anti-invasion, anti-metastasis</td>
<td>Runx2, MMPs</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Apoptosis</td>
<td>MMP-2, BCL-2, BAX</td>
</tr>
<tr>
<td></td>
<td>Anti-proliferation</td>
<td>Fen1, Nrf-2, BPA proliferation, Skp2, p27</td>
</tr>
<tr>
<td></td>
<td>Cell cycle transition</td>
<td>miR-19</td>
</tr>
<tr>
<td></td>
<td>Apoptosis</td>
<td>BAX, BCL-2, caspase-3, cytochrome c</td>
</tr>
<tr>
<td></td>
<td>Anti-proliferation</td>
<td>Akt/GSK-3beta/cyclin D1 signaling</td>
</tr>
<tr>
<td></td>
<td>Cell cycle transition</td>
<td>PCNA, cyclin D1</td>
</tr>
<tr>
<td>Furanodiene</td>
<td>Cell cycle transition, anti-invasion, anti-metastasis</td>
<td>MMP-9</td>
</tr>
<tr>
<td>Genipin</td>
<td>Apoptosis</td>
<td>BAX, BCL-2, caspase-3, JNK, p38MAPK, UCP2, ROS</td>
</tr>
<tr>
<td>Ginsenoside</td>
<td>Apoptosis, cell cycle transition</td>
<td>MDM2</td>
</tr>
<tr>
<td>Lycopene</td>
<td>Anti-proliferation</td>
<td>Skp2</td>
</tr>
<tr>
<td></td>
<td>Anti-metastasis, anti-invasion</td>
<td>ERK/Akt signaling pathway</td>
</tr>
<tr>
<td></td>
<td>Apoptosis</td>
<td>BAX, caspase-9, cyclin D1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Akt/mTOR signaling pathway</td>
</tr>
<tr>
<td>Morin</td>
<td>Anti-invasion, anti-metastasis</td>
<td>Akt pathway signaling</td>
</tr>
<tr>
<td>Nexitrine</td>
<td>Apoptosis, cell cycle transition</td>
<td>Cyclin D1, cdk2</td>
</tr>
<tr>
<td>Phytoestrogens</td>
<td>Apoptosis, anti-angiogenesis,</td>
<td>ROS/p38 MAPK pathway, BCL-2, promoters I.3/I1</td>
</tr>
<tr>
<td>Pterostilbene</td>
<td>Apoptosis, anti-proliferation</td>
<td>BAX</td>
</tr>
<tr>
<td>Retinoid</td>
<td>Apoptosis, anti-proliferation</td>
<td>ER/HER2 signaling</td>
</tr>
<tr>
<td>Sauchinone</td>
<td>Apoptosis</td>
<td>VEGF, cyclin D1, BCL-2, caspase-3</td>
</tr>
<tr>
<td>Tehranolide</td>
<td>Anti-proliferation, cell cycle transition</td>
<td>PI3K/Akt/cyclin D1 pathway, ROS, cytochrome c, BAX, BCL-2</td>
</tr>
<tr>
<td>Ursolic acid</td>
<td>Apoptosis</td>
<td>FoxM1, cyclin D1/CDK4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAX, BCL-2, cytochrome c</td>
</tr>
</tbody>
</table>
2.8.2. *Tinospora cordifolia* in inhibition of cancer

Various studies have demonstrated the presence of various groups of phytochemicals in *T. cordifolia* extracts which inhibits cellular proliferation in various *in vitro* models and also show antineoplastic [Jagetia and Rao, 2006], antitumor [Adhvaryu et al., 2008; Singh et al., 2005; Thippeswamy and Salimath, 2007], anti-angiogenesis [Leyon and Kuttan, 2004; Thippeswamy et al., 2008] and anti-metastatic activity [Shilpa et al., 2015] in various *in vivo* models.

Individual parts or whole *T. cordifolia* plant have been reported to improve the immune system and anti-oxidant properties in various *in vitro* and *in vivo* models [StanelyMainzen Prince and Menon, 2001]. Previously, the anti-inflammatory effect of *T. cordifolia* was seen on paw edema model in rats induced by carrageenan [Patgiri et al., 2014]. Several reports indicate anti-inflammatory activity of the decoction [Sharma and Singh, 1981], alcohol extract [Wesley et al., 2008], water extract of the stem of *T. cordifolia* [Pendse et al., 1977; Utpalendu et al., 1999] and *T. cordifolia* extract has been proved to possess protective effect against asthmatic inflammation and oxidative stress [Tiwari et al., 2014]. Epicatechin from *T. cordifolia* stem extract showed antioxidant property [Pushp et al., 2013].

2.8.3. Pyrrole

Pyrrole a five-membered nitrogen-containing aromatic heterocyclic compounds that is commonly found in natural products, pharmaceuticals and bioactive molecules. Pyrrole is widely known as a biologically active scaffold which possesses a diverse nature of activities. The combination of different functional groups in a pyrrole ring system has led to the formation of pharmacologically active compounds. Pyrrole containing analogs are considered as a potential source of biologically active compounds that contains a significant set of advantageous properties and can be found in many natural products. Pyrrole derivatives have diverse applications in therapeutically active compounds including antibiotics, fungicides, anti-inflammatory drugs, cholesterol reducing drugs, antitumor agents and many more. Due to the diversity of these analogs in the therapeutic response profile, many researchers have been working to explore this skeleton to its maximum potential against several diseases or disorders [Bhardwaj et al., 2015].

It is interesting to note that in recent years a significant number of marine natural products possessing a pyrrole ring system have been isolated, characterized, and bioassayed [Montaser and Luesch, 2011]. Dolastatin-10, Bryostatin-1 and Cryptophycin-52 of marine natural product origin are currently in Phase II clinical trials for the treatment of various forms of cancer [Newman and Cragg, 2004]. Roseophilin and Prodigiosins are the natural products of pyrrolo-alkaloids that exhibit a broad range of activity. Fürstner, 2003 reported that roseophilin exhibits higher cytotoxicity against several cancer cells. In vivo studies suggest that prodigiosins acts synergistically with cyclosporine A which is a reference immunosuppressive agent. A series of synthesized aryl pyrroles were evaluated in vivo and in vitro for antitumor activity among which LB42908 was found as a highly active antitumor agent, and currently undergoing preclinical studies as inhibitor of RAS farnesyl transferase (FTase) [Lee et al., 2001].
Cdc7 serine/threonine kinase is a key regulator of DNA synthesis in eukaryotic organisms. Cdc7 inhibition through siRNA or prototype small molecules causes p53 independent apoptosis in tumor cells while reversibly arresting cell cycle progression in primary fibroblasts. This implies that Cdc7 kinase could be considered a potential target for anticancer therapy. A new chemical class of 5-heteroaryl-3-carboxamido-2-substituted pyrrole derivative was synthesized by introducing a variety of substituents at position 2 of pyrrole ring. The compound with phenyl substituent at 2 position of pyrrole and 2-amino-4- pyrimidin-4-yl chain at position 5 represented a novel prototype Cdc7 kinase inhibitor [Menichincheri et al., 2010].

Similarly, Siddiqui et al., [2015] synthesized compound that showed promising anticancer activity against human leukemia cell line (HL-60) by MTT assay. The presence of [30, 50-dimethylpyrazole-1-yl] carbonylmethoxy moiety attached at 3b position was responsible for this enhanced activity [Siddiqui et al., 2015]. The ultrasound assisted and bismuth nitrate catalyzed ecofriendly route was developed to synthesize a series of novel N substituted pyrrole derivatives. Some synthesized pyrrole derivatives were highly cytotoxic against some cancer cell lines. When compared with normal hepatocytes in vitro, these compounds were selectively cytotoxic against hepatic cancer cell lines. The study suggested that N-substituted pyrrole exhibits different mechanism of cytotoxicity as compared to other polyaromatic derivatives [Bandyopadhyay et al., 2012].

The novel 1H-pyrrolo [2, 3-b] pyridine derivatives were synthesized for the treatment of diffuse malignant peritoneal mesothelioma (DMPM). Compounds consistently reduced DMPM cell proliferation by inducing a caspase dependent apoptotic response with a concomitant reduction of the expression of active Thr34-phosphorylated form of the anti-apoptotic protein surviving [Carbone et al., 2013]. The Structure activity
relationships investigation studies were performed on the C2-position of PBD monomer antitumor agents. Compound delayed tumor growth in HCT-116 (bowel) human tumor xenograft model. The study demonstrated that the cytotoxicity and DNA binding affinity of PBD conjugates can be enhanced by introducing C2-quinolinyl substituent. Moreover, this compound delayed tumor growth in HCT-116 colon cancer xenograft model without causing weight loss or other adverse effects, which suggested that C2-aryl PBD monomers could be used as potential agents in the treatment of human disease [Antonow et al., 2010].

Anticancer activity of compounds could be attributed to the induction of caspases activation dependent apoptosis through loss of mitochondrial membrane potential, followed by release of cytochrome-c and increase in BAX level, and decrease in BCL-2 level [Fang et al., 2010].

2.9. EAT model

Ehrlich ascites carcinoma (EAT) otherwise known as Ehrlich ascites tumor (EAT) is derived from mouse mammary carcinoma and one of the most accepted transplantable tumor models [Ozaslan et al., 2011]. EAT is widely used in oncology laboratories because of its rapid proliferation rate, 100% malignancy, high transplantable capability, no-regression, shorter life span, and lack of tumor-specific transplantation antigen. EAT cells grow in suspension in the peritoneal cavity of mice and they do not adhere to the synthetic surface in vitro. The effusion, which contained neoplastic cells that are proliferated after injection of tumor cells into the peritoneal cavity, is referred to as the “ascites” [Kaleoğlu and İşli, 1977]. In 4 or 6 days after passage, the ascites fluid is formed and a total of 5 or 12 ml ascites fluid is accumulated. Frequently, tumor virulence increases via repetitious passages, while the proliferating rate of such tumors increases gradually. However, the differentiation gradually disappears, while the cells get free growth control mechanisms,
gain hetero-transplantability and in the end, they are converted to the ascites’ form. Several studies have reported that following i.p., transplantation of $3 \times 10^6$ EAT cells into a mouse, the number of cells increased exponentially in the 9 days and they switch over from the exponential phase to the plateau phase starting from the 9th and 10th day [Cragg and Newmann, 1999].

EAT has a resemblance with human tumors which are the most sensitive to chemotherapy due to the fact that it is undifferentiated and that it has a rapid growth rate. Due to the resemblance, researchers use this model for the antineoplastic study [Ozaslan et al., 2009]. Although there are a lot of reports of phytochemical studies, approximately only 10% of the 250,000 complex plant species have been investigated for their chemical and pharmacologically important compounds. Nonetheless, the search of new toxic agents from natural sources has been conducted in collaboration with scientists worldwide [Cragg and Newmann, 1999].

2.10. 2, 4- Dinitrophenol

![Figure 2.5 Structure of 2,4- Dinitrophenol.](image)

2, 4-Dinitrophenol (DNP) is an organic compound with the molecular formula HOC$_6$H$_3$(NO$_2$)$_2$ (Fig. 2.5). DNP has historically been used in the manufacture of food coloring, clothing dyes, explosives, agricultural herbicides, insecticides and fungicides [Yen and Ewald 2012; Lee et al., 2014]. The compound is known to uncouple mitochondrial
Chapter 2: Review of literature

oxidative phosphorylation and was used as an anti-obesity agent early in the past century. Because of its potentially fatal adverse effects, including hyperthermia, cataract, agranulocytosis, hepatotoxicity, nephrotoxicity and cardiotoxicity, the compound was subsequently banned by the United States Food and Drug Administration [Colman, 2007]. Banning DNP sale for human consumption protects the general public but DNP is still sold mostly via internet sales. DNP is purchased and used by determined users who are not dissuaded from experimenting with DNP based on health threats. However, the popularity of DNP as a slimming aid has resurfaced again in recent years [Petrócsi et al., 2015]. DNP is used by bodybuilders and extreme dieters for its fat burning properties through inhibiting efficient energy (ATP) production in cells [Tewari et al., 2009]. Through uncoupling mitochondrial oxidative phosphorylation by facilitating proton transport across the mitochondrial membrane, DNP leads to rapid consumption of energy without generating ATP and consequently, to increased fat metabolism. DNP is sold in different names like dinosan, dnoc, solfo Black, nitrophen, aldifin and chemox. Some internet sites have DNP available in bulk quantities, allowing users to purchase kilograms of DNP powder or hundreds/thousands of DNP-containing tablets [Grundlingh et al., 2011]. Water contamination due to spillage of DNP during transportation or from factories and industries leads to toxic injuries on exposure [Jiukun et al., 2011]. Moreover, adults and children from farming communities are vulnerable to toxic injuries from the chemical DNP present in pesticides and insecticides [Macnab and Fielden, 1998]. The classic phenomena noticed by overdose of phenol-based product, DNP is a combination of hyperthermia, tachycardia, diaphoresis and tachypnea [Bartlett et al., 2010]. In animal studies, DNP has been shown to be teratogenic, mutagenic and carcinogenic; including developmental and reproductive toxicity has been reported [Takahashi et al., 2009]. Owing to acute DNP
toxicity and preceding death, the patient is often profoundly hyperthermic associated with methaemoglobinaemia, a disorder with higher level of methemoglobin [Hsiao et al., 2005]. Methemoglobin is a form of abnormal hemoglobin that contains iron in ferric (Fe$^{3+}$) form rather than the usual ferrous (Fe$^{2+}$) form. Ferric (Fe$^{3+}$) iron has a decreased ability to bind oxygen but the ferrous iron has an increased affinity for bound oxygen. The binding of oxygen to methemoglobin results in an increased affinity of oxygen to the three other heme sites (that are still ferrous) within the same tetrameric hemoglobin unit. This leads to an overall reduced ability of the red blood cell to release oxygen to tissues [Ash-Bernal et al., 2004].