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ANTICANCER ACTIVITY OF GUGGULSTERONE IN HUMAN LEUKEMIC CELLS

BY

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ABSTRACT

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Title: Anticancer Activity of Guggulsterone in Human Leukemic Cells

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Leukemia is a group of blood cancers that is characterized by the uncontrolled proliferation of hematopoietic cells and their progressive accumulation within the bone marrow (BM) and secondary lymphoid tissues. The main cause of leukemia remains unclear, with a combination of genetic and environmental factors involved. Current treatment options have several limitations with major side effects, mainly related to high toxicity. In that respect, alternative forms of treatment are required to effectively manage and treat leukemia patients. Natural products have been shown to effectively treat several types of human cancers. These natural products include plants and other natural substances. Once natural product that has shown promising anti-cancer properties and has been found to possess cancer chemopreventive and therapeutic potential in a number of cancer cell lines is the plant polyphenol Guggulsterone (GS), which is extracted from the gum resin of the *commiphora mukul* tree. Nevertheless, to date, few studies have investigated the effects of GS in the treatment of leukemia. In this respect, this study focuses on the efficiency of GS in the treatment of leukemia. In this study, we demonstrated that guggulsterone inhibited the viability of human leukemia cells by inducing apoptosis through activation of the intrinsic mitochondrial pathway. Anti-tumour activity of guggusterone has been found to be associated with activation of caspase cascade, upregulation of the proapoptotic proteins (Bax and Bid) and downregulation of the antiapoptotic proteins (Bcl-2, Bcl-xL, xIAP, cIAP-1, cIAP-2 and survivin). Furthermore, guggulsterone was found to regulate STAT3 signaling

pathway. Another specific objective of this study was to exploit the anticancer potential of guggulsterone in combination with the existing chemotherapeutic approved platinum drug cisplatin. Our results revealed that guggulsterone acts synergistically with cisplatin to inhibit the viability of leukemia cells and improved the chemosensitivity of cisplatin. Our results demonstrate that guggulsterone could serve as a potent natural anti-cancer agent that may serve as a promising effective treatment option for leukemia alone or in combination therapies. Our findings serve as a basis for investigating novel regimens to prevent or delay the development of platinum resistance and overall improve the treatment of leukemia.

DEDICATION

This thesis is dedicated to:

The sake of Allah,

My family in my homeland Egypt, and

Qatar University; my second magnificent home.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

1.1.Leukemia overview

Leukemia is a group of blood cancers that is characterized by the uncontrolled proliferation and abnormal development of non-functional leukocytes and their precursors, causing their accumulation in various body organs and tissues; mainly blood, bone marrow, lymph node, and spleen (Dohner, Weisdorf, & Bloomfield, 2015). The crowding of leukemia cells causes the expulsion of the normal hematopoietic system, leading to serious complications, such as anemia, thrombocytopenia, and immunodeficiency.

Blood cancers represent the 11th and 10th most frequent cause of cancer occurrence and death worldwide, respectively, with more than 350000 new leukemia cases and 265000 leukemia deaths in 2012 (Ferlay, Soerjomataram et al., 2013). Generally, leukemia survival, incidence, and mortality patterns are highly dependent on diagnosis, prognosis, and natural history of neoplasms arising from the haemopoietic stem or progenitor cells found in the bone marrow (Rodriguez-Abreu, Bordoni, & Zucca, 2007).

Leukemia is typically categorized according to rate of progression and blast phenotype into two broad categories; acute and chronic. Acute leukemia is generally characterized by quick progress and high number of immature undifferentiated cells (blasts); whereas, chronic leukemia is characterized by slow progress and lower number of blasts. Leukemia is further classified as either myelogenous or lymphocytic depending on the cell lineage involved (Swerdlow, International Agency for Research on, & World Health, 2008; Weinberg, 2013). The four major subtypes are acute lymphoblastic leukemia (ALL), chronic lymphoblastic leukemia (CLL), acute myeloid

leukemia (AML), and chronic myeloid leukemia (CML) (Swerdlow, International Agency for Research on et al., 2008; Weinberg, 2013). ALL is considered to be the most common cancer among children and the leading cause of cancer-related death among people under the age of forty (Kaatsch, 2010), while the other subtypes are more frequent among adults.

For the majority of patients, the causes of leukemia and its subtypes are unclear, this is in part due to the diversity of the abnormalities and the different risk factors involved. However, genetic background interacting with environmental factors, including exposure to high doses of radiation or carcinogenic chemicals, parental occupational exposures, and infections have been linked to increased risk of leukemia (Chokkalingam & Buffler, 2008).

1.2. Current treatment options

Treatment options differ depending to the type of leukemia and other factors, including age and general health. Currently, chemotherapy, stem cell transplantation, and targeted therapies are available. Other treatment options, such as surgery, radiation therapy, leukapheresis, or treatment with monoclonal antibodies may be considered under certain conditions. The regeneration of cancer tissues and chemo resistance towards therapies have been a major challenge owing to the intricate cellular machinery that cancer cells exhibit. This has encouraged clinicians and scientists to investigate the multi-focused use of existing drugs to address chemotherapy limitations in alternate or safer treatment strategies.

1.3. Natural compounds as anti-cancer agents

Many studies have demonstrated the pharmacological activities of natural compounds, including antioxidant (Kandikattu, Rachitha et al., 2017), antimicrobial

(Harakeh, Khan et al., 2017), antidiabetic (Liu, Zhai, Han, & Yin, 2016), and anti-inflammatory effects (Mbiantcha, Almas, Dawe, Faheem, & Sidra, 2018). Most recently, natural compounds have gained a considerable interest among researchers due to their cytotoxic activity or apoptotic effects on cancer. Most importantly, some natural compounds were shown to exhibit selective prominent cytotoxic effects in cancerous cells while causing very low intrinsic toxicity in normal cells (Abdal Dayem, Choi et al., 2016). In addition, recent studies have shown that natural compounds can restore the sensitivity of cancer cells to conventional chemotherapeutic drugs (Xiucheng Li, Huang et al., 2013). A combination therapy that joins traditional chemotherapy with natural compounds, is now considered a new innovative approach for overcoming multidrug resistance and cell toxicity.

Many medicinal plants have been studied as treatment for cancer. In fact, most of the natural compounds currently available as pharmaceutical products isolated from plant extracts have proven to exhibit anti-tumor properties in vivo and in vitro. Plants make a diversity of compounds that can be divided into primary metabolites and secondary metabolites. Primary metabolites include all metabolic pathways that are crucial to the plant's survival, like growth and development of the plant. However, the secondary metabolites are not essential to the plant's survival, but they have several biochemical functions. These compounds protect plants, and they have a role; they are pollinator attractants and serve as chemical defenses against microorganisms, bacteria, viruses, insects and higher predators (Fürstenberg-Hägg, Zagrobelny, & Bak, 2013).

1.4. (Z)-Guggulsterone

Z-Guggulsterone (GS), a plant polyphenol derived from the resin (guggulu) of the plant *commiphora mukul* tree, has been shown to inhibit cancer cells from further

growth via inducing apoptotic cell death in a variety of cancer cell lines (Y. Gao, Zeng et al., 2014; Jiang, Xiao et al., 2013; Kong, He et al., 2015; S. V. Singh, S. Choi, Y. Zeng, E. R. Hahm, & D. Xiao, 2007; S. V. Singh, Zeng et al., 2005; W. C. Wang, Uen et al., 2012). Nevertheless, to date, scarce number of studies have explored the role of GS in leukemia treatment. In this respect, this research focuses on the effectiveness of GS in the treatment of leukemia. Here, GS significantly induced apoptosis and cell cycle arrest in leukemia cell lines. These results provide evidence that GS contain bioactive compounds that could be used in the treatment of leukemia.

1.5. Aims and objectives

The first aim of this study was to determine the efficacy of GS alone, and in combination with the chemotherapeutic agent cisplatin in leukemia cells. The second aim was to elucidate the mechanism of action of GS using human leukemia cell lines.

The specific objectives for this study were to:

A. To determine whether GS treatment of leukemia cells suppresses cell proliferation.
B. To determine whether GS can induce cell cycle arrest and apoptosis in leukemia cells
C. To determine the what signaling pathways is involved in GS mediated apoptotic cell death in leukemia cells
D. To determine whether GS mediated apoptosis occur through mitochondrial (intrinsic) or and/or death receptor (extrinsic)
E. To determine if GS can enhance the anticancer efficacy of traditional chemotherapeutic agents when used in combination.

1.6. Significance

The collective burden of cancer and current cancer therapeutics imply that there is a critical need for developing new treatments that are more selective, effective, and cost-effective. Many traditional chemotherapeutics are unable to selectively target cancer cells and are known to induce apoptosis in non-cancerous healthy cells and cause major toxicities in various organs. The purpose of this study is to develop a leukemia therapeutic agent of natural origin, as a safe alternative for killing leukemia cells. Many oncologists are rightfully hesitant to promote the usage of guggulsterone alongside chemotherapeutics due to a lack of reproducible scientific validation. The scientific studies carried out in this thesis will provide evidence allowing for the introduction of guggulsterone as an efficacious natural agent for treating leukemia. In doing so, we will be able to provide a safer and cheaper complementary treatment for leukemia to improve patient prognosis.

2. LITERATURE REVIEW

2.1. Cancer

Cancer is a major and global health concern and despite many advances in cancer treatment approaches, cancer continues to remain one of the leading causes of death around the world. Cancer can be described as an illness which happens when cells in a specific part of the body begin to grow out of control. The body is made up of trillions of cells. It is a dangerous disease caused mainly by environmental factors. Such factors caused genes to mutate and express important cell regulatory proteins. It can also be defined as a genetic disease mainly caused by environmental factors. Cancer causing agents are typically found in foods, beverages, air, sunlight or chemicals.

2.1.1. Incidence of cancer

Cancer is the second leading disease after heart affiliated diseases in the world (H. Wang, Naghavi et al., 2016). It is estimated that approximately 40% all men and women in the United States would be diagnosed with cancer throughout their lifetime (Arem & Loftfield, 2017).

In 1996, there were approximately 10 million new cases of tumors all over the world (Bray, Ferlay et al., 2018). It is predicted that by 2020, there would be over 20 million new cases of cancer and over twelve million deaths ("Centers for Disease Control and Prevention," 2018). Moreover, 30% of cancer deaths have been estimated in developed countries while 70% has been estimated in developing countries (Mathers, Shibuya, Boschi-Pinto, Lopez, & Murray, 2002).

In Canada, 1 in 4 diagnosed patients from of 206,200 projected cancer incidences were expected to succumb to the disease in 2018 (Siegel, Miller, & Jemal, 2017). In the United States, 1,762,450 new cancer cases are projected to be diagnosed, with

approximately 1 in 3 diagnosed patients expected to succumb to the disease in 2019 (Siegel, Miller, & Jemal, 2019). Cancer is a disease characterized by uncontrolled cell growth and proliferation resulting from evasion of regulatory mechanisms in cells.

2.1.2. Classification of cancer

There are over 200 types of cancers would wide (Chambers, Groom, & MacDonald, 2002). They are categorized according to either the tissue of origin or the location at which they first develop. They are grouped into six major categories, namely carcinoma, sarcoma, myeloma, leukemia, lymphoma, and mixed types.

Approximately 80-90% of cancers are referred to as "carcinomas" which are the cancers that start in cells that make up the epithelial tissues such as the lungs, kidneys, liver, skin, intestines and mammary glands. Cancers that originate from non-epithelial cells like mesoderm cells such as bone, muscle, fat, blood vessels, cartilage, or other soft or connective tissues are known as "sarcomas". Cancers of glandular tissues such as breast tissue are called "adenocarcinomas" (Ying, Dey et al. 2016). Cancer of the blood is known as leukaemia; also known as "liquid cancer", while cancer in lymphatic system is referred to lymphoma or "solid cancer". Based on the anatomical location; cancer can be names for example; lung or breast cancers. Cancers may exhibit distinct features based on their origin. For instance, skin cancer has many characteristics that make it exhibit different characteristics from lung cancer.

2.1.3. Causes of cancer

The development of cancer is known to occur over a period of time with various contributing factors that are involved in tumorigenesis through damaging genetic material, which is normally repaired or triggers senescence or elimination of the cell depending on extent of damage. These contributing factors are known to be a

combination of environmental (such as; include unhealthy lifestyle, exposure to environmental carcinogens) and genetic factors (genetic predisposition).

This multi-step process involves the accumulation of multiple mutations required for its progression from in situ dysplasia to malignant tumor (Loeb, Springgate, & Battula, 1974). Causes of cancer are mainly classified as internal and external factors. Internal factors include genetic factors, ageing and hormonal imbalances; which are regarded as three main internal factors causing cancer. External factors include environment, lifestyle (such as smoking and alcohol consumption), diet, toxic chemicals, radiation exposure, viruses are also found to be linked to cause various cancers (Stewart & Kleihues, 2003).

Most of human cancers result from exposure to environmental carcinogens; which include both natural and man-made chemicals, radiation, and viruses. In fact, it is estimated that 30% of cancers are be attributed to tobacco use and diet, which implies that cancer could be prevented if only strong measures were implemented. Carcinogens may be divided into a number of classes (Table-1). “Primary carcinogens” (also known as genotoxic carcinogens) which react with nucleic acids and directly affect cellular constituents, nongenotoxic carcinogens (also called epigenetic carcinogens) which function to induce tumor formation by mechanisms other than the direct modification or damage to DNA, and finally “procarcinogens”, which are those that require metabolic activation to induce carcinogenesis. The molecular diversity of the cancer-initiating compounds can range from metals to complex chemicals, with variations in potency, suggesting that more than one mechanism is involved in carcinogenesis.

Carcinogens in the diet that trigger the initial stage include moulds and aflatoxins (for example, in peanuts and maize), nitrosamines (in smoked meats and other cured

products), rancid fats and cooking oils, alcohol, additives and preservatives. A combination of foods may have a cumulative effect, and when incorrect diet is added to a polluted environment, smoking, UV radiation, free radicals, lack of exercise, and stress, the stage is set for DNA damage and cancer progression.

Table 1. Types of carcinogens

S.No	Types of carcinogens	Carcinogens agent
1	Genotoxic carcinogen Primary, direct-acting alkylating agents	Dimethylsulfate, ethylene imine, β-propiolactonel
2	Procarcinogens I. Polycyclic aromatic hydrocarbons II. Nitrosamines III. Hydrazine IV. Inorganic	Benzo[a]pyrene Dimethylnitrosamine 1,2-Dimethylhydrazine Cadmium, plutonium
3	Epigenetic carcinogens I. Promotors II. Solid state III. Hormones IV. Immunosuppressants V. Cocarcinogens	Phorbol esters, saccharin, bile acids Asbestos, plastic Estrogens Purine analogues Catechol
4	Unclassified agents Peroxisome proliferators	Clofibrate, phthalate esters

2.1.4. Carcinogenesis

The conversion of normal cells to cancerous cells is known to occur through a multi-stage process and a long period of time. This multi-stage process involves; initiation, promotion, and progression. Initiation; the first stage, is when the cancer-producing substances; commonly referred to as "carcinogens" react with the DNA inside the cells. This stage may remain dormant, and the individual may only be at risk of developing cancer at later stages. The second stage is referred to as "promotion" which is believed to occur very slowly over a period of time ranging from several months to years. During this stage, a change in diet and lifestyle is vital to reduce the chance of developing cancer later in life. Progression; the third and final stage, involves the spread of the cancer to other areas, at this stage, diet may have less of an impact.

2.2.Normal Regulatory Cellular Pathways

2.2.1. Normal haematopoiesis

Haematopoietic stem cells (HSCs) are found in abundance in the bone marrow (BM) and are capable of generating different types of blood cells, commonly classified as either lymphoid or myeloid lineage (Motonari Kondo, 2010). The lymphoid lineage includes T cells, B cells and natural killer (NK) cells. While the myeloid lineage includes erythrocytes, granulocytes, monocytes, and megakaryocytes (M. Kondo, Wagers et al., 2003; Weissman, 2000). Additionally, HSC have been shown to produce cell types that are not directly related to the haematopoietic process, such as osteoclasts (Gabrilovich & Nagaraj, 2009; Mehrotra, Williams, Ogawa, & LaRue, 2013). T lymphocytes play a key role in antigen-recognition; their proliferation, differentiation and effector roles necessitate the recognition of specific antigens, necessary to assist

other types of cells, such as B cells and NK cells (Alegre, Frauwirth, & Thompson, 2001). B lymphocytes respond to foreign antigens, which are recognized by membrane bound IgM, after binding, they divide and differentiate into plasma cells, which produce antibodies with the same specificity as the original B-cell (Bernasconi, Traggiai, & Lanzavecchia, 2002). B-cells are produced in the BM prior to release into the circulation where they migrate to secondary lymphoid tissues where they differentiate into terminally differentiated plasma cells (Calame, 2001). In healthy individuals, the proliferation, differentiation and release of cells from the BM is a highly controlled process, if any defects arise, it may trigger a variety of human diseases, including leukemia (Greim, Kaden et al., 2014).

2.2.2. Cell cycle

The cell cycle is a sequence of events by which a cell grows and divides. It is a strictly controlled process that involves five main phases: G0, G1, S, G2 and M (Nurse, 2000). When cells leave a state of quiescence (G0), they enter a first gap phase (G1). Many signaling pathways feed into the cell cycle machinery in G1, also, all prerequisites for proper S-phase progression are being checked during this phase. When cells leave the G1 phase, they enter S phase (synthesis); at which DNA synthesis occurs. During S phase the quantity of chromosomal DNA is replicated to create exactly two identical chromosomes. Subsequently, a second gap phase (G2) where they prepare to divide before entering mitosis (M), at which nuclear division occurs, splitting into two identical daughter cells (Vermeulen, Van Bockstaele, & Berneman, 2003). The process of mitosis occurs in two stages; mitosis and cytokinesis. During mitosis the chromosomes in the nucleus of the parent cell divide into two daughter cells. In cytokinesis, the cytoplasm of the parental cell divides into two daughter cells

(Vermeulen, Van Bockstaele et al., 2003).

2.2.2.1.Regulation of cell cycle

Cell cycle regulation is essential to ensure maintaining faithful segregation of genetic material and thus enabling the natural development and maintenance of multicellular organisms (Vermeulen, Van Bockstaele et al., 2003). Failure to manage these processes can contribute to genomic instability that can lead to cancer. There are a variety of checkpoints during the cell cycle that track and control the pacing and progression throughout the cell cycle, these checkpoints occur at the Gi/S phase boundary, in S phase, and during G2/M phases. These checkpoints ensure that the correct sequence of events in particular the phase of cell cycle is completed successfully before a new phase is initiated (Harbour & Dean, 2000; Meeran & Katiyar, 2008). The cell cycle is regulated by a number of protein-controlled feedback processes. Two groups of proteins involved in the control of the cell cycle are cyclins and cyclin-dependent kinases (Cdks).

2.2.2.1.1. Cyclins and kinases

Cyclins are a family of proteins that regulate the progression of the cell cycle. Cyclins activate kinases by binding to them, forming cyclin dependent kinases (CDKs). Once activated, they activate or inactivate other molecules by phosphorylation. CDKs also play a role in other important cellular functions, including transcription, mRNA processing, and neuronal differentiation (Bendris, Lemmers, & Blanchard, 2015; Lim & Kaldis, 2013; Vermeulen, Van Bockstaele et al., 2003).

2.2.2.1.2. Cyclin-dependent kinase inhibitors (CDKIs)

Cell cycle is negatively regulated by cyclin-dependent kinase inhibitors (CDKIs) which are small proteins that inhibit CDKs (Meeran & Katiyar, 2008). There

are two major families of CDKIs: the INK4 (inhibitor of CDK4) family and the Cip/Kip (kinase inhibitor protein) family (Meeran & Katiyar, 2008; Vermeulen, Van Bockstaele et al., 2003). The INK4 family includes four members: pl6^{INK4a}; pl5^{INK4b}; pl8^{INK4c} and pl9^{INK4d}, which are inhibitors of CDK4. Each member of the INK4 family is encoded by a unique gene and there are 15- to 19-kDa polypeptides that share approximately 40% homology with one another (Vermeulen, Van Bockstaele et al., 2003). The Cip/Kip family is made up of three members: p21$^{cip1/waf1}$(Gu, Turck, & Morgan, 1993; Xiong, Hannon et al., 1993)/ p27kipl(Toyoshima & Hunter, 1994) and p57^{kip2}(Lee, Reynisdottir, & Massague, 1995; Matsuoka, Edwards et al., 1995), which inhibit the activities of most CDKs (Vermeulen, Van Bockstaele et al., 2003). The members of the Cip/Kip family share sequence homology at the N-terminal domain which allows them to bind to both the cyclin and CDK. Dis-regulation of molecules controlling the cell cycle plays an important role in the cancer pathogenesis (Meeran & Katiyar, 2008). For instance, alterations within the CDKIs, including pl6 and p21 have been found in many human cancers (G. D. Chen, Qian et al., 2017; Dai & Grant, 2003; Guo, Huang, & Ji, 2017; Shariat, Tokunaga et al., 2004; Vallmanya Llena, Laborda Rodriguez et al., 2006; F. L. Wang, Yang, Liu, Qin, & Jin, 2017; Zhang, Li, & Lu, 2015; Zinczuk, Zareba et al., 2018). Because CDK dis-regulation is reported in most human tumor cells, pharmacological CDK inhibition has become an attractive approach regarding non-genotoxic and mechanism-based therapies in oncology (Fischer & Gianella-Borradori, 2003).

2.2.2.1.3. Retinoblastoma (Rb*)*

Retinoblastoma (Rb) is a tumor suppressor; commonly referred to as the master regulator of cell cycle, it plays a vital role in inhibiting cell cycle progression and

blocking cell growth (Burkhart & Sage, 2008; Giacinti & Giordano, 2006). The Rb family includes a group of proteins collectively referred to as 'pocket proteins' consisting of three members: Rb/pl05, pl07 and Rb2/pl30. The function of RB in cell cycle regulation is mediated through its interaction with E2F; a group of transcription factors that regulate the cell cycle (Burkhart & Sage, 2008; Dimova, Stevaux, Frolov, & Dyson, 2003; Qian, Luckey, Horton, Esser, & Templeton, 1992; Stevaux & Dyson, 2002). The Rb protein binds to E2F family members, repressing gene transcription needed for S-phase entry and progression. Thus, loss of Rb function may result in cell cycle dis-regulation contributing to both cancer initiation and progression. Chromosomal mutations and Rb inactivation are often seen as key components in the development of human cancers, including; lung (Wikenheiser-Brokamp, 2006), brain (Jacks, Fazeli et al., 1992), and liver (Hui, Li, Makuuchi, Takayama, & Kubota, 1999) cancers, in addition to different types of leukemia (Krug, Ganser, & Koeffler, 2002), particularly acute myeloid leukemia (Tang, Yeh et al., 1992).

2.2.3. Programmed cell death (apoptosis)

Apoptotic cell death is a homeostatic mechanism for maintaining cell populations in tissues through the processes of development and aging (Elmore, 2007). Apoptosis is also considered a defense mechanism that occurs during immune reactions or when cells are damaged (Norbury & Hickson, 2001). Apoptosis can be initiated by a wide variety of stimuli, both physiologic and pathologic, including: cell stress and DNA damage, nevertheless, not all cells will necessarily undergo apoptosis in response to the same stimulus (Elmore, 2007). In most situations, cells undergoing apoptosis display a very characteristic and similar features of classical morphological changes (Hacker, 2000). These morphological changes include cell shrinkage; membrane

blebbing; nuclear DNA fragmentation; chromatin condensation and formation of apoptotic bodies (Saraste & Pulkki, 2000). Apoptotic bodies then are immediately phagocytosed by neighboring macrophages and thus clear cancerous and pre-cancerous cells without eliciting any inflammatory response, reducing the likelihood of tissue damage induced by excessive autoimmune responses (Hofmann, de Vos et al., 2001; Ren & Savill, 1998).

There are essentially two major signaling pathways for initiating apoptosis; which are greatly sophisticated and involve an energy-dependent cascade of events: (i) intrinsic apoptotic pathway (mitochondrial mediated), and (ii) extrinsic apoptotic pathway (death receptor mediated) (Figure 1). Both pathways are regulated with enzymatic caspase activation, and also molecular systems, including bcl-2/bax and Fas/Fas ligand (Ashkenazi, 2008; Brady, 2003; Martin, 2006).

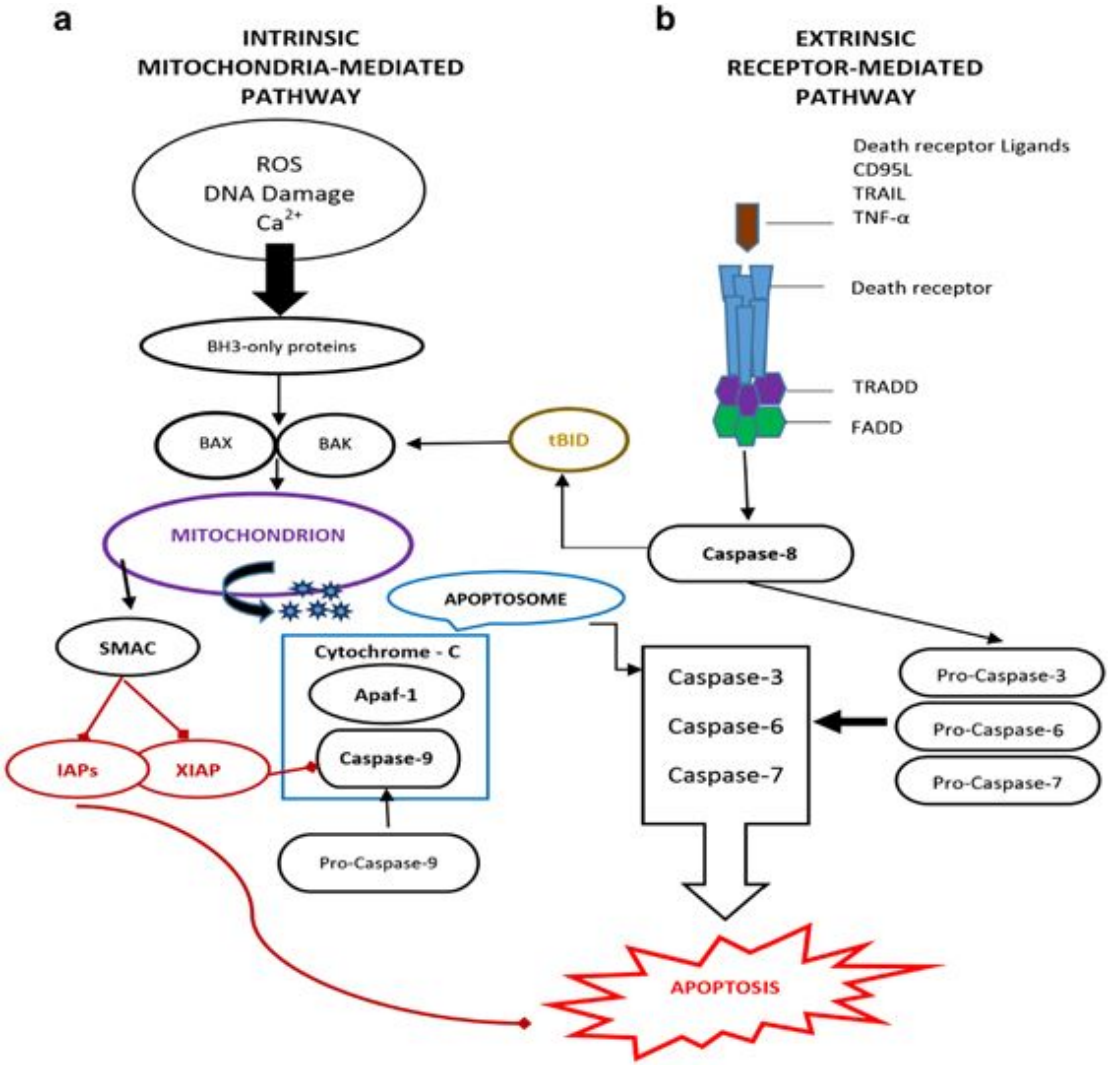

Figure 1. The mitochondria-mediated intrinsic (a) and death receptor-mediated extrinsic (b) pathway. Apaf-1, apoptotic protease activating factor 1; FADD, Fas-associated death domain; TRADD, TNFR-associated death domain protein.

2.2.3.1.The intrinsic pathway (mitochondrial pathway)

In the intrinsic or 'mitochondrial mediated' pathway (Figure 1a), apoptosis is activated by pro-apoptotic stimuli from inside the cell, such as the deprivation of certain growth factors; cytokines; hormones; oxidants; hypoxia; lack of nutrients; or infections

lead to initiation of death programs, this in turn causes activation of apoptosis (Elmore, 2007; Saelens, Festjens et al., 2004; Yip & Reed, 2008). Normally, these stimuli activate the expression of potent regulatory protein members of the Bcl-2 family that act by stimulating BH3-only family proteins which activates the pro-apoptotic effectors BAX and BAK. These pro-apoptotic effectors can alter the permeability of the outer mitochondrial membrane causing mitochondrial outer membrane permeabilization (MOMP). MOMP causes to the release of proteins from the intermembrane space into the cytosol. Some of these proteins are considered 'innocent bystanders' and do not elicit any particular cellular response following their release from the intermembrane space. However, others, such as cytochrome c and second mitochondria-derived activator of capsase/Direct inhibitor of apoptosis-binding protein Smac/DIABLO, promote cell death by activating the caspase-dependent mitochondrial pathway (Garrido, Galluzzi et al., 2006; Hill, Adrain, Duriez, Creagh, & Martin, 2004; Saelens, Festjens et al., 2004). Following the release of cytochrome C from the intermembrane space, it binds and forms a complex with dATP, apoptotic protease activating factor 1 (APAF-1) as well as the procaspase-9, forming a multimeric complex referred to as the "apoptosome" (Hill, Adrain et al., 2004; Khan, Wagner, Yule, Bhanumathy, & Joseph, 2006), which functions as a platform for caspase-9 activation which in turn activates downstream effector caspases in the cytosol and executes apoptosis. Cytochrome c is crucial for the formation of the apoptosome and activation of caspase 9, thus, without MOMP and the release of cytochrome c from the intermembrane space, caspase 9 activation and subsequent activation of downstream effector caspases does not occur (P. Li, Nijhawan et al., 1997). The protein Smac/ DIABLO also functions to promote caspase activation following its release from the intermembrane space, although via a

different mechanism from cytochrome c. Smac/DIABLO promotes caspase activation by neutralizing inhibitor of apoptosis (IAP) proteins, that function by binding to and preventing the activities of caspase 9 and effector caspases in the cytosol, eventually promoting caspase 9 and effector caspase activation and executing apoptosis (Du, Fang, Li, Li, & Wang, 2000). Although MOMP-induced caspase activation is absolutely necessary for mitochondrial apoptotic cell death, inhibition of caspase activity in the presence of MOMP can still lead to non-apoptotic forms of cell death, due to loss of mitochondrial function. Thus, the factors that control the integrity of the OMM literally control the life and death of the cell. Additionally, factors such as mitochondrial dynamics, mitochondrial bioenergetics and mitochondrial cristae remodeling also contribute to MOMP and the overall health status of the mitochondria.

2.2.3.1.1. Regulation of the intrinsic pathway

2.2.3.1.1.1.Bcl-2-family

The Bcl-2-family includes a number of evolutionarily-conserved proteins that are structurally similar; they play an important role in the regulation of the intrinsic apoptotic pathway by controlling mitochondria membrane permeability and the release of pro-apoptotic factor: cytochrome c. Bcl-2 family proteins consists of members that can be divided into three main groups based on their structures and intracellular functions (Figure 2): those that promote apoptosis (Bak, Bax, Bcl- rambo, Bcl-xs, BOK/Mtd); those that inhibit apoptosis (Bcl-2, Bcl-xl, Bcl-w, Mcl-1, Bcl-10, and Bcl-2 related protein Al); and the pro-apoptotic BH3-only proteins, which bind to and regulate the anti-apoptotic Bcl-2 proteins (Bad, Bid, Bik/NBK, Bim/Bod, Bmf, Hrk/DP5, Noxa and Puma/BBC3; several other proteins may also be included in this

subfamily, depending on the definition of what constitutes a BH3 domain) (Cory & Adams, 2002; Elmore, 2007; Yip & Reed, 2008). The ratio between anti- and pro-apoptotic family members controls whether or not cells will undergo apoptosis (Elmore, 2007).

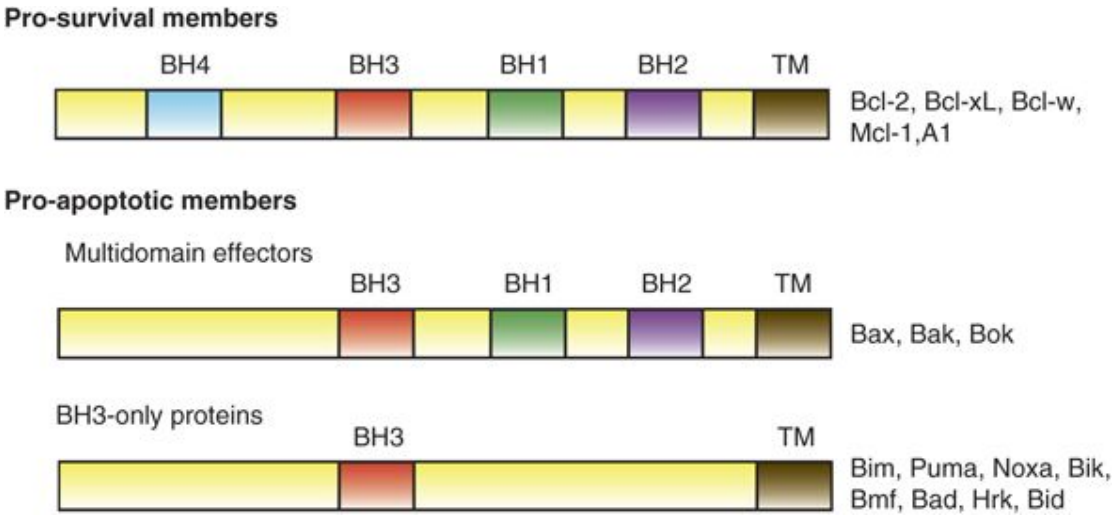

Figure 2. The Bcl-2 family is made up of proteins that contain conserved functional Bcl-2 Homology domains. The family can be subdivided into pro-survival and pro-apoptotic proteins. The latter can be further divided into multidomain effectors and BH3-only proteins. The transmembrane domain (TM) is not found in some of the BH3–only proteins.

2.2.3.1.1.2.Non-Bcl2 family proteins

Apart from the Bcl-2 family proteins, apoptotic inducing factor (AIF), endonuclease G and caspase-activated DNAse (CAD) are other proteins that have a pro-apoptotic role and are released from the mitochondria at the late stages of apoptosis. AIF and endonuclease G translocate from the mitochondria and move to the nucleus

and to induce large-scale DNA fragmentation and chromatin condensation (Joza, Susin et al., 2001; X. Li, Marani et al., 2001). AIF and endonuclease G both function in a caspase-independent manner (Enari, Sakahira et al., 1998). In addition, caspase 3 acts by cleaving the inhibitor of caspase-activated DNAse (ICAD) and allows CAD to cleave the DNA between individual nucleosomes at random points (Larsen, Rampalli et al., 2010). On the contrary, there are some proteins which act as inhibitors, including intracellular IAPs which regulate caspase activity by binding and inhibiting the activation of pro-caspases and the activity of mature caspases (Khan, Wagner et al., 2006). Some of these inhibitors are X-linked inhibitor of apoptosis protein (XIAP), Baculoviral IAP repeat-containing protein 3 (CIAP2) and Survivin (van Loo, van Gurp et al., 2002). SMAC/Diablo and HtrA2/Omi are described to stimulate apoptosis by inhibiting the activity of IAP (van Loo, van Gurp et al., 2002). SMAC/Diablo proteins are activated by the effect of apoptosis inducing factor (AIF) (Khan, Wagner et al., 2006)

2.2.3.2.The extrinsic pathway (death receptor pathway)

The extrinsic or 'death receptor mediated' pathway of apoptosis is initiated by external factors from outside the cell, when an apoptotic signal is received through binding of pro-apoptotic ligands such as: Apo2L (apoptosis ligand 2); Apo3L (apoptosis ligand 3); TRAIL (tumor necrosis factor-related apoptosis-inducing ligand); Fas Ligand and TNF a to their specific pro-apoptotic membrane death receptors such as DR4 (death receptor 4); DR5 (death receptor 5); Fas and TNF Rll (Rodriguez et al, 2005; Martin, 2006; Ashkenazi, 2008). After binding, the death domain of each receptor reacts with Fas-associated death domain (FADD) causing the recruitment and formation of a complex referred to as "death-inducing signalling complex (DISC)"

(Ashkenazi & Dixit, 1998; Locksley, Killeen, & Lenardo, 2001). Formation of the DISC can be initiated independently by each through the recruitment of the the adapter FADD and activating the initiator caspases-8 and -10 (Ashkenazi, 2008; Elmore, 2007; Kischkel, Lawrence et al., 2000). In turn, activated caspase 8 activates the executioner caspase 3, which is essential for DNA fragmentation and chromosomal condensation. The cross linking between extrinsic and intrinsic pathway occurs when the active caspase 8 activates the pro-apoptotic member Bid (Bcl-2 interacting domain), to trigger the release of cytochrome C (Elmore, 2007; Fadeel & Orrenius, 2005; Martin, 2006). Cellular FADD-like interleukin-ip-converting enzyme inhibitory protein (c-FLIP) may inhibit this pathway.

2.2.3.3.The tumor suppressor protein p53

The tumor suppressor protein p53 can directly or indirectly control the expression or release of pro-apoptotic and anti-apoptotic proteins, as well as proteins that control mitochondrial membrane permeability and thus, can modulate the release of mitochondrial proteins in intrinsic apoptosis (Elmore, 2007; Hofseth, Hussain, & Harris, 2004). In addition, within the intrinsic pathway p53 increases the expression of APAF-1. Intriguingly, p53; also known to regulate extrinsic apoptosis by increasing expression of cellular death receptors, such as DR5. p53 can be induced because of many signals, for example; telomere shortening; DNA damage; oncogene activation and over expression of tumor suppressor genes (Miura et al, 2004). Thus, it is apparent that p53 is an important tumor suppressor protein at the crossroads of cellular stress response pathways. It can initiate cell-cycle arrest, DNA repair, chromosomal segregation, cellular senescence and differentiation (Hofseth, Hussain et al., 2004). The different functions of activated p53 are complicated and highly dependent on the co-

expression of multiple factors which vary according to cell type as well as by the severity and persistence of conditions of cell stress and genomic damage (Hanahan and Weinberg, 2011). It has been shown previously that the loss of p53 in myeloid progenitor cells is associated with a high risk of AML development, since p53 plays a role in cell proliferation regulation by limiting normal HSCs self-renewal (Zhao et al, 2010). Apoptosis mechanisms are highly complex and associated by complex cascades of intracellular events that include activation of pro-apoptotic Bcl-2 family, member of the caspase family and several nucleases.

Deregulation within one or more of the normal regulatory pathways, including; cell proliferation, differentiation, and apoptosis could cause cancerous cells to develope, proliferate and evade death which, which may eventually lead to cancer. These features constitute the hallmarks of cancers which are required for malignant transformation.

2.3. Hallmarks of Cancer

Six different properties of cancer cells have been attributed to the progression of cancer in humans. (Hanahan & Weinberg, 2011) primarily described these six main hallmarks of cancer as follows: (1) evading growth suppressors; (2) resisting apoptosis; (3) sustaining proliferative signaling; (4) angiogenesis induction; (5) enabling replicative immortality; and (6) invasion and metastasis activation (Hanahan & Weinberg, 2011; Pietras & Ostman, 2010). In 2011, they added four more hallmarks: (1) abnormal metabolic pathways; (2) immune system evasion; (3) unstable DNA and chromosome abnormalities, and (4) inflammation (Figure 3). Targeting of these hallmarks may lead to the development of improved cancer therapeutics (Hanahan & Weinberg, 2011), and may act as targets for new leukemia therapies, such as resisting cell death, evading

growth suppressors, and sustain proliferation signaling, which will be discussed further in the following subsections.

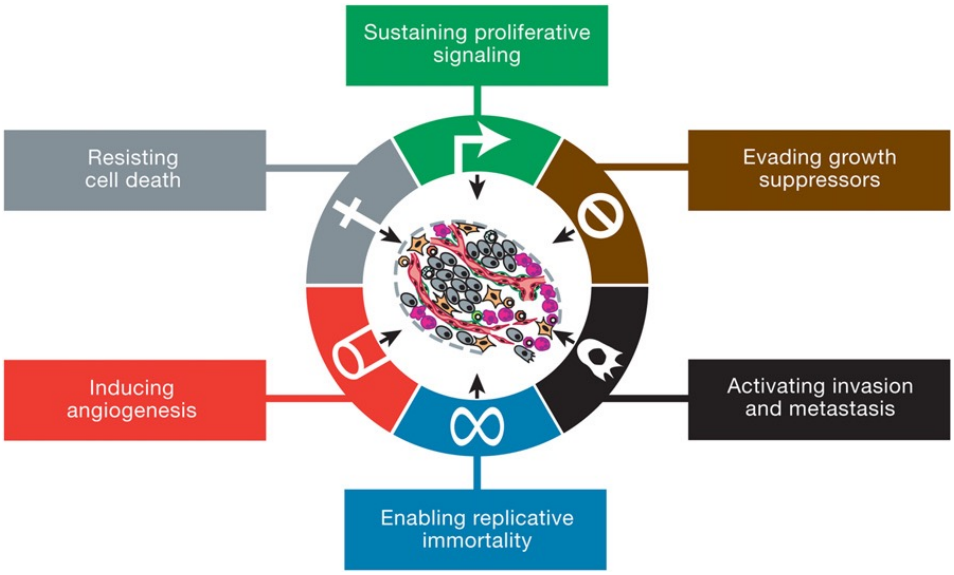

Figure 3. The Hallmarks of Cancer.

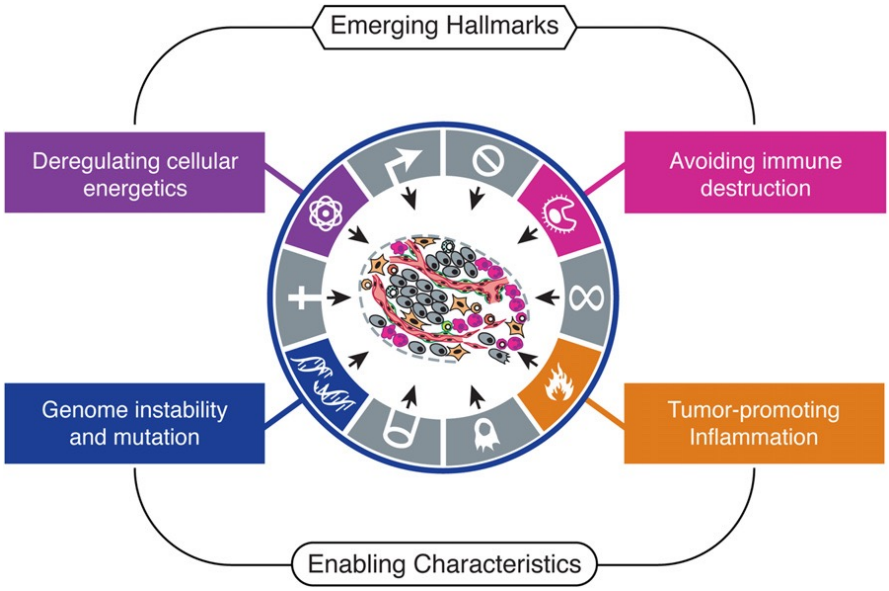

Figure 4. Emerging Hallmarks and Enabling Characteristics.

2.3.1. Resisting cell death

Resisting programmed cell death plays a key role in cancer cell survival. The most common mechanism by which cancer cells resist apoptosis is by modulation of p53 either though gene deletions or mutations (Bouillet and Strasser, 2002; Junttila et al, 2009). Alternative mechanisms include the over expression of anti-apoptotic proteins such as Bcl-2, and Bcl-xLor by down regulating pro-apoptotic proteins such as Bax. Modulation of extrinsic apoptosis is also seen via decreased expression of death receptors (Hanahan & Weinberg, 2011).

2.3.2. Evading growth suppressors

Inside normal healthy cells, proliferation is strictly regulated by CDK and CDKI. Specifically, during Gi stage which is an important checkpoint where anti--proliferation signals develop their activity to avoid more cell proliferation such as Rb. The Rb protein actively inhibits cell passage though the restriction (R) point in the Gi phase and decides if the cell should proceed or not (Sherr and McCormick, 2002; Burkhart and Sage, 2008). Thus, cancer cells with mutated Rb remove this gatekeeper and allow ongoing cell proliferation. On the other hand, p53 functions to arrest the cell cycle once DNA damage is detected and acts as a central regulator of apoptosis (Hanahan & Weinberg, 2011; Hofseth, Hussain et al., 2004). p53 mutation is important in a number of cancers and is linked to poor prognosis in chronic lymphocytic leukemia (CLL) patients (Zenz et al, 2010), and associated with the aggressive forms of Acute myeloid Leukemia (AML) (Zaho etal, 2010).

2.3.3. Sustaining proliferation signaling

Cell signaling is a highly controlled mechanism in normal cells; however, this regulation is impaired during cancer (Hanahan & Weinberg, 2011). One of the essential

actions of cancer cells is their capacity to proliferate in an uncontrollable manner, which is accomplished by sending signals to normal cells to provide cancer cells with more growth factors; increasing growth factor production by the cancerous cells and increasing the number of growth factor receptors expressed on the cell surface contributing to auto proliferation stimulation (Hanahan & Weinberg, 2011).

2.3.4. Standard therapies of cancer

In the 1600BC, the stomach cancer was treated using boiled barley mixed with dates. On the other hand, the cancer of the uterus was treated using a concoction of fresh dates with a mixture of pig brains introduced in the vagina. Radiotherapy was not developed until 1900AD. Chemotherapy was then later introduced in 1945AD as a treatment. In this day and age, there are various ways in which a cancer patient could be treated. The conventional methods include radiotherapy, psychosocial support, chemotherapy, and surgery. These methods are considered according to the level of cancer the patient is having. They are supposed to either cure the patient or to prolong their life ensuring improved and quality life. Early detection of cancer before it spread to other parts of the body can be easily contained. If a tumor is discovered when it has already spread, it will be difficult to cure the patient. It is in this stage that treatment strategies are put in place to ensure that the life of the patient is prolonged until the patient's body cannot take in no more and passes on.

2.4.Leukemia

Leukemia is a complex form of blood malignancy characterized by the uncontrolled proliferation of haematopoietic cells and progressive accumulation of these cells within the BM and secondary lymphoid tissues, which can spill over into the peripheral blood

and other organs. This accumulation prevents the production of other vital normal blood cells such as red blood cells and platelets resulting in anemia, bleeding and immunodeficiency (Buffler, Kwan, Reynolds, & Urayama, 2005).

2.4.1. Leukemia statistics

Leukemia is a major problem worldwide affecting many people each year. It is estimated that more than a quarter of million people died from leukemia in 2008. Leukemia is the ninth most common cancer death in the UK and the fifth in the USA. There were approximately 9,918 new cases of leukemia in the UK in the years between 2014 and 2016, and about 4,712 deaths from leukemia in the UK in 2016 ("Leukaemia (all subtypes combined) statistics," 2015). In the USA, there were approximately 23,540 death in 2012 ("Cancer Facts & Figures 2012 | American Cancer Society," 2012). ln France, during the period 2000 to 2004, leukemia and lymphoma was the most frequent cancer accounting for approximately 41% of the total diagnosed malignancies (Lacour, Guyot-Goubin et al., 2010). In 2013, around 4,800 people are expected to be diagnosed with leukemia and more than 23,000 people expected to die from leukemia in USA (Siegel et al, 2012)

Generally, leukemia occurs with varying frequencies at different ages and is more common in adults than children. Overall leukemia is more common in males than females. Leukemia causes about one-third of all cancer deaths in children. Acute lymphoblastic leukemia (ALL) is the most common type in children. More than 50% of all leukemia diagnosed in children are ALL and the risk for getting it, is highest in children under 5 years old (Cancer Research UK, 2013). In the USA, ALL accounted for 74% of new leukemia cases in children (Leukemia & Lymphoma Research, 2013). AML and CLL are the most common type in adults (Cancer Research UK, 2013;

Leukemia & Lymphoma Research, 2013). In USA, more than 14,500 new cases of AML were reported in adult and 10,370 deaths from this blood malignancy (American cancer society, 2013). Moreover, according to Leukemia & Lymphoma Research around 2200 people are diagnosed with AML in the UK annually.

In Leukemia relative survival rate vary according to patient's age at diagnosis, gender, and type of leukemia (Leukemia & Lymphoma Research, 2013). The death rates from leukemia are very low in people under the age of 50 years old but rise dramatically in the over 60's (Cancer Research UK, 2012). In the USA, the mortality rate from chronic myeloid leukemia (CML) showed a decrease in 2005 comparing to the five years before while the AML showed a significant increase in the same period of time (Radich, 2010). In the UK, between 2005 and 2009, 44% of people survived from leukemia for at least five years post diagnosis (Cancer Research UK, 2013). Moreover, mortality rates for both men and women from leukemia shows a very gradual decline between the late 1970's and 2008 in the UK (Leukemia & Lymphoma Research, 2012). Therefore, an improved understanding of the pathogenesis of leukemia and the development of novel drugs is essential to improve the prognosis of leukemia patients.

2.4.2. Leukemia classification

A number of classifications for hematopoietic blood malignancies have been identified such as French-American-British (FAB), Revised European-American Lymphoma (REAL) and 2001 and 2008 World Health Organization (WHO) classifications. These classification systems were based on the identification of distinct tumors using clinical features, immunophenotype, genetic information, molecular and morphological investigation of the peripheral blood (PB) and BM specimens (Gralnick et al, 1977; Neame et al, 1989 and Vardiman et al, 2009). In addition, these classifications have

some similarity, for example the diagnosis of AML is usually dependant on the level of blast cells as in the blood or bone marrow smears, however, the most important difference between the 2001 WHO system and FAB classifications for the diagnosis of this disorder was the lowering of the blast threshold from 30% to 20% in the PB or BM smears (Vardiman et al, 2002). According to the 2008 WHO classification, the name myeloid includes all granulocytic cells (neutrophil, eosinophil, basophil), monocytic/macrophage, erythoid, megakaryocytic and mast cell lineages, while the lymphoid malignancies include T-cell and B-cell lineages (Vardiman, 2010). Moreover, within the updated WHO classification the definitions of some well-established disorders such as CLL, plasma cell neoplasm's and Waldenstrom macroglobulinemia (WM) were improved (Morgan, 2003; Owen et al, 2003; Hallek et al, 2008; Vardiman, 2010) and tumor location and age groups, such as the elderly and children were linked to the incidence of certain types of Leukemia (Campo et al, 2011). Leukemia classification is important to determine the cellular maturation degree and origin of the Leukemia cells from where they were originated which is an important tool to determine therapeutic choices and patient's survival.

However, in this thesis the four main classification of Leukemia were used to describe the types of cells studied as most research studies related to Leukemia treatment are based on this classification and by using such classification, we can identify stage of cellular maturation and the origin of the leukemia cells. Within this thesis the classification system divides Leukemia into four large groups, including: acute, which is a rapidly progressing disease that results in the accumulation of immature cells in the bone marrow and blood, or Chronic, which progresses more slowly and allows partially mature cells to form. These can be either myeloid or lymphoid origin (Peacock, 2000;

Leukemia & Lymphoma Research, 2012). If Leukemia begins in early forms of myeloid cells, including red blood cells, platelets or white blood cells (but not T, B, lymphocytes, or NK cells) this is considered as myeloid Leukemia. Conversely, in lymphoblastic Leukemia the cancer starts in early form of lymphocytes in bone marrow. Therefore, there are generally four types of Leukemia commonly termed: AML, ALL, CML and CLL (Peacock, 2000). The first three of these types arise from HSCs whilst CLL is derived from mature B lymphocytes.

2.4.3. Causes of leukemia

The exact cause of leukemia remains unclear (Greaves, 1997; Buffler and Kwan, 2005; Buffler et al, 2005; Eden, 2010). Leukemia is thought to have multifactorial causes which involve interaction between different aspects originating from the environmental as well as human genetics (Buffler et al, 2005). However, there are a number of factors involved which increase the chance of leukemia developing.

Exposure to Certain Chemicals: The risk of leukemia may be increased by exposure to certain chemicals. For example, long-term exposure to high levels of benzene is a risk factor for AML (Weng et al, 2004; Buffler and Kwan, 2005; Rossi et al, 2000). In addition, Smoking is known to be linked to cancers of the mouth, lung, and throat, but studies have shown that it can also affect cells which do not come into direct contact with smoke (Weng et al, 2004; Buffler and Kwan, 2005).

Age and Gender: Leukemia is more common in men than women and the risk of getting leukemia increases with age, but the reasons for this are not clear (Weng et al, 2004).

Family History: Most cases of leukemia are not thought to have a strong genetic linkage; however, having a close relative in some types of leukemia enhances the risk

of getting the disease. For example, in identical twins, where one developed AML before they were a year old increased the risk of the second twin developing AML demonstrating a genetic linkage (Amigou etal, 2011).

High-Dose Radiation Exposure: Exposure to high-dose radiation (for example as being a survivor exposed to the Hiroshima and Nagasaki atomic bombs or nuclear factor accident) is known to be associated with increased risk of leukemia such as CLL (Weng et al, 2004; Buffler and Kwan, 2005; Rossi et al, 2000).

Viral infection: Epstein-Bar virus (EBV) and human T-cell lymphoma leukemia virus (HTLV-1) have been implicated in the development of leukemia. The EBV is a herpesvirus that can inhibit B-lymphocytes and nasopharyngeal cells. The HTLV-1 virus is closely associated with T-cell lymphocytic leukemia found in Japan, Africa (Lackritz, 2000).

Genetic abnormalities: Genetic defects and abnormalities are key risk factors associated with the incidence of certain types of leukemia (Buffler and Kwan, 2005; Rossi et al, 2000; Amigou et al, 2011). A number of syndromes that result from genetic mutations present at birth seem to increase the risk of leukemia. These include; bloom syndrome, blackfan-diamond syndrome, and fanconi anemia. Down syndrome and trisomy 8 which are caused by chromosome problem present at birth are also linked to raise the risk of leukemia (Weng et al, 2004; Buffler and Kwan, 2005; Rossi et al, 2000).

2.4.4. Leukemia pathogenesis

A transforming event in a hematopoietic stem cell causes genetic alterations which result in neoplastic hematopoietic disorders. Based on the occurring mutation, cells can go through different routes: a) mature abnormally and die prematurely, resulting in cytopenia and myelodysplasia or myelodysplastic syndromes; b)

Proliferate, and eventually crowding out normal hematopoietic cells, resulting in acute leukemia; or c) fail to die, and thus accumulate until they reach high numbers in blood, causing chronic leukemia. These are not necessarily mutually exclusive events, moreover, due to genomic instability, cells in chronic leukemia and myelodysplastic syndromes can mutate further and progress to acute leukemia.

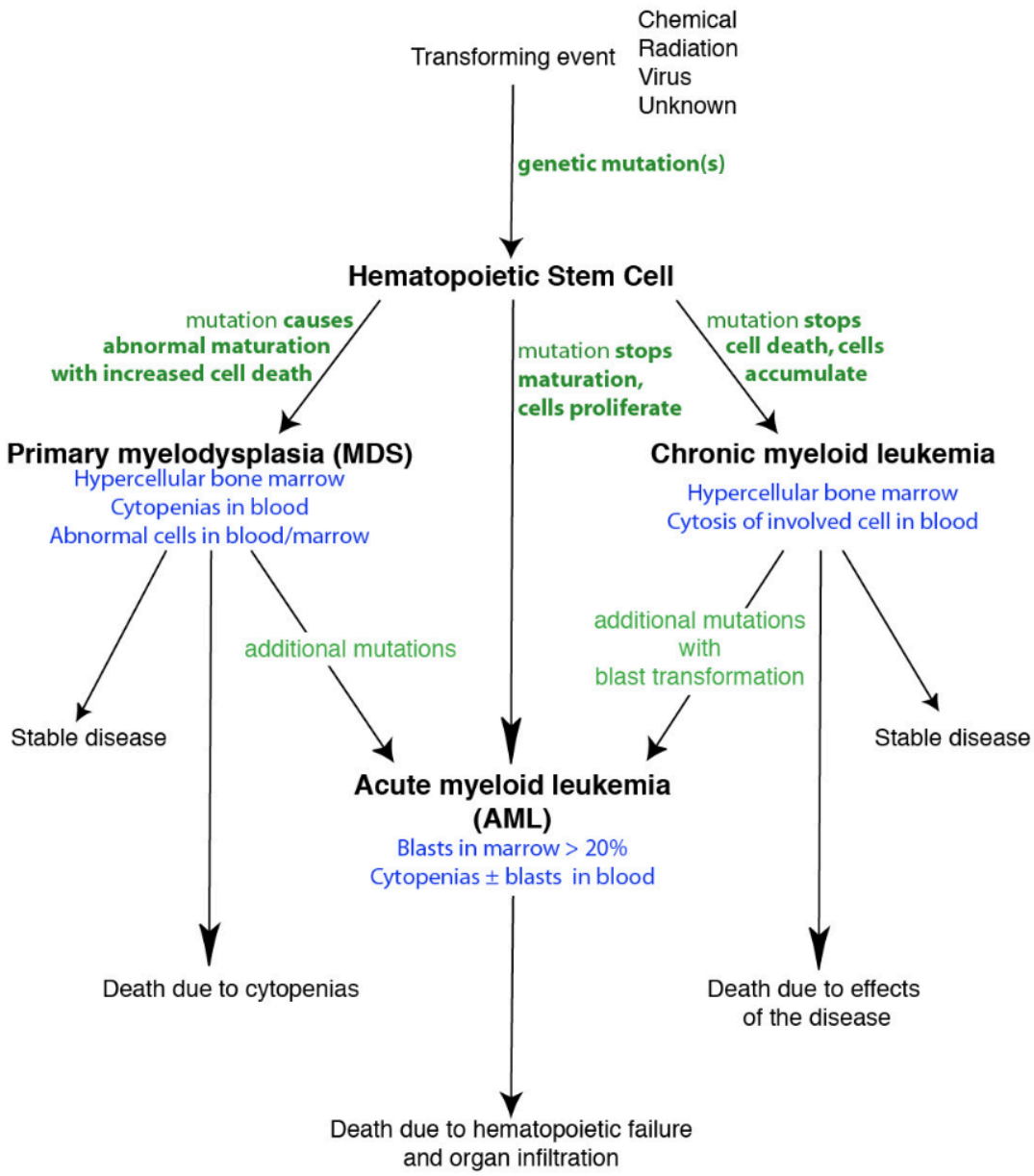

Figure 5. Pathogenesis of leukemia syndromes.

2.4.5. Diagnosis of leukemia

Because many types of leukemia show no obvious symptoms early in the disease, leukemia may be diagnosed incidentally during a physical exam or as a result of routine blood testing. If a person appears pale, has enlarged lymph nodes, swollen gums, an enlarged liver or spleen, significant bruising, bleeding, fever, persistent infections, fatigue, or a small pinpoint rash, the doctor should suspect leukemia. A blood test showing an abnormal white cell count may suggest the diagnosis. To confirm the diagnosis and identify the specific type of leukemia, a needle biopsy and aspiration of bone marrow from a pelvic bone will need to be done to test for leukemia cells, DNA markers, and chromosome changes in the bone marrow. Important factors in leukemia include the age of the patient, the type of leukemia, and the chromosomal abnormalities found in leukemia cells and bone marrow.

2.4.6. Leukemia treatment

The goal of current Leukemia treatments is to kill the leukemia cells and allow normal cells to form in the BM. The treatment depends on a number of factors such as histologic type of leukemia, its stage, and prognostic features (patient's age and overall health) (Appelbaum etal, 2006). Chemotherapy isthe most common treatment for most types of Leukemia and their side effects vary depending on the type of therapy. Bone marrow transplantation is a relatively straightforward medical procedure. Diseased or damaged bone marrow can be replaced by donated bone marrow from healthy patient, which helps treat, and often cure, many serious, life- threatening conditions, including Leukemia (Laughlin et al, 2004). This choice of Leukemia treatment provides a very high rate of success (Laughlin et al, 2004).

Radiotherapy can be used as part of the preparation for BM transplantation to destroy

the cancerous BM with the Leukemia cells using very high level of energy (Walch et al, 2013). Radiation may also use as single therapy for different types of malignant diseases such as breast cancer (Radiation et al, 2006). In addition, growth factor treatments such asgranulocyte colony stimulating factor (G-CSF) may be used to stimulate the BM to synthesis more blood cells to decrease the risk of infection that is generated as a result of low level of WBC following the chemotherapy (Dombret et al, 1995; Lowenberg et al, 2003). The survival rate in patients with AML was about 9% higher following the treatment with chemotherapy plus G-CSF than patients who did not receive G-CSF (Lowenberg et al, 2003).

Targeted cancer therapy is a type of medical treatment designed to treat cancer by blocking the growth and spread of cancer by interfering with specific target molecules involved in tumor growth and progression rather than simply interfering with rapidly dividing cells (e.g. chemotherapy). Targeted therapies can cause cancer cell death by inducing apoptois or arresting cell cycle.

2.4.6.1.Targeting cell cycle

The cell cycle is a series of events which allow the cell to grow and proliferate. Important parts of the cell cycle mechanism are the CDKs which, when activated, provide a means for the cell to move from one phase of the cell cycle to the next (Section 1.1.2.1) (Schwartz and Shah 2005). The CDKs are regulated positively by cyclins and regulated negatively by naturally occurring CDKIs (Section 1.1.2.1.1 and 1.1.2.1.2). Cancer is characterized by a dysregulation of the cell cycle such that cells overexpress cyclins or do not express CDKIs and thus, continue to undergo unregulated cell growth (Section 1.1.2.1). The cell cycle also works to protect the cell from DNA damage. Therefore, cell cycle arrest is a survival mechanism which gives the cancer cells the

opportunity to repair their damaged DNA. Recently, in clinical trials are a series of targeted agents that directly in inhibit CDKs, inhibit unrestricted cell growth, and induce growth arrest (Schwartz and Shah 2005).

2.4.6.2.Targeting apoptosis

To date, many of the crucial players in the system of apoptosis regulation are identified and can be targeted by therapeutic strategies which include Bcl-2 proteins, caspases, and death receptors. Therefore, identification of the major regulators increases research into developing therapeutic approaches to intervene either in a pro- or anti- apoptotic direction (Ghobrial et al, 2005). Another approach is to classify the agents as those that target the extrinsic pathway, intrinsic pathway, or the proteins regulating apoptosis (Ghobrial et al, 2005). In addition, some drugs aimed to control apoptosis indirectly by targeting protein kinases, transcriptional factors, phosphatases, proteasomes and cell surface receptors (Ghobrial etal, 2005).

2.4.7. Leukemia treatment

The treatment of leukemia is in constant flux, evolving and changing rapidly over the past few years. The basic strategy in treating to eliminate all detectable disease by using cytotoxic agents. Commonly used cytotoxic drugs include metabolic analogs, gluco- corticoids, asparaginase, anthracyclines, and vincristine. Chemotherapeutic agents kill rapidly dividing cells, thus slowing down and stopping the spread of cancerous cells.

Most leukemia treatment regimens are composed of chemotherapy with or without radiotherapy. Chemotherapy for leukemia usually involves giving several drugs together as a regimen. Because each drug has its own drawbacks, a combination

of drugs may make the cells more vulnerable to treatment.

Chemotherapy for leukemia patients is usually given orally, in pill form or administered intravenously. In some cases, chemotherapy drugs may be delivered intrathecally, directly through the cerebrospinal fluid, by lumbar puncture (also called a spinal tap), or through a special device under the scalp. Some of the currently approved drugs and combination regimens for leukemia are listed in table 2.

Table 2. Approved chemotherapeutic drugs and combination regimens for leukemia.

ALL	**AML**	**CLL**	**CML**
Approved drugs			
Arranon (Nelarabine)	Arsenic Trioxide	Acalabrutinib	Bosulif (Bosutinib)
Asparaginase Erwinia chrysanthemi	Cerubidine (Daunorubicin Hydrochloride)	Alemtuzumab	Bosutinib
Asparlas (Calaspargase Pegol-mknl)	Cyclophosphamide	Arzerra (Ofatumumab)	Busulfan
Besponsa (Inotuzumab Ozogamicin)	Cytarabine	Bendamustine Hydrochloride	Busulfex (Busulfan)

ALL	AML	CLL	CML
		Bendeka	
	Daunorubicin	(Bendamustine	
Blinatumomab	Hydrochloride	Hydrochloride)	Cyclophosphamide
	Daunorubicin		
	Hydrochloride and		
Blincyto	Cytarabine	Calquence	
(Blinatumomab)	Liposome	(Acalabrutinib)	Cytarabine
	Daurismo		
Calaspargase	(Glasdegib	Campath	
Pegol-mknl	Maleate)	(Alemtuzumab)	Dasatinib
Cerubidine			
(Daunorubicin			
Hydrochloride)	Dexamethasone	Chlorambucil	Dexamethasone
	Doxorubicin	Copiktra	Gleevec (Imatinib
Clofarabine	Hydrochloride	(Duvelisib)	Mesylate)
Clolar	Enasidenib		Hydrea
(Clofarabine)	Mesylate	Cyclophosphamide	(Hydroxyurea)
	Gemtuzumab		
Cyclophosphamide	Ozogamicin	Dexamethasone	Hydroxyurea
	Gilteritinib		Iclusig (Ponatinib
Cytarabine	Fumarate	Duvelisib	Hydrochloride)

ALL	AML	CLL	CML
		Fludarabine	
Dasatinib	Glasdegib Maleate	Phosphate	Imatinib Mesylate
	Idamycin		
Daunorubicin	PFS (Idarubicin	Gazyva	Mechlorethamine
Hydrochloride	Hydrochloride)	(Obinutuzumab)	Hydrochloride
			Mustargen
	Idarubicin		(Mechlorethamine
Dexamethasone	Hydrochloride	Ibrutinib	Hydrochloride)
Doxorubicin	Idhifa (Enasidenib		Myleran
Hydrochloride	Mesylate)	Idelalisib	(Busulfan)
Erwinaze			
(Asparaginase			
Erwinia		Imbruvica	
Chrysanthemi)	Ivosidenib	(Ibrutinib)	Nilotinib
Gleevec (Imatinib		Leukeran	Omacetaxine
Mesylate)	Midostaurin	(Chlorambucil)	Mepesuccinate
Iclusig (Ponatinib	Mitoxantrone	Mechlorethamine	Ponatinib
Hydrochloride)	Hydrochloride	Hydrochloride	Hydrochloride
	Mylotarg	Mustargen	
Inotuzumab	(Gemtuzumab	(Mechlorethamine	Sprycel
Ozogamicin	Ozogamicin)	Hydrochloride)	(Dasatinib)

ALL	AML	CLL	CML
	Rubidomycin (Daunorubicin Hydrochloride)		Synribo (Omacetaxine Mepesuccinate)
Imatinib Mesylate	Rydapt (Midostaurin)	Obinutuzumab	
Kymriah (Tisagenlecleucel)	Tabloid (Thioguanine)	Ofatumumab	Tasigna (Nilotinib)
Marqibo (Vincristine Sulfate Liposome)	Thioguanine	Prednisone	
Mercaptopurine	Tibsovo (Ivosidenib)	Rituxan (Rituximab)	
Methotrexate	Trisenox (Arsenic Trioxide)	Rituxan Hycela (Rituximab and Hyaluronidase Human)	
Nelarabine	Venclexta (Venetoclax)	Rituximab	
Oncaspar (Pegaspargase)		Rituximab and Hyaluronidase Human	
Pegaspargase	Venetoclax	Treanda (Bendamustine Hydrochloride)	

ALL	AML	CLL	CML
Ponatinib Hydrochloride	Vincristine Sulfate Vyxeos (Daunorubicin Hydrochloride and Cytarabine Liposome) Xospata (Gilteritinib Fumarate)	Truxima (Rituximab) Venclexta (Venetoclax) Venetoclax Zydelig (Idelalisib)	
Prednisone			
Purinethol (Mercaptopurine)			
Purixan (Mercaptopurine)			
Rubidomycin (Daunorubicin Hydrochloride)			
Sprycel (Dasatinib)			
Tisagenlecleucel			
Trexall (Methotrexate)			
Vincristine Sulfate			
Vincristine Sulfate Liposome			

ALL	AML	CLL	CML
Approved Drug Combinations			
Hyper-CVAD	ADE	CHLORAMBUCIL-PREDNISONE CVP	

2.4.8. Platinum-based drugs

Over the past 30 years, platinum-based drugs, such as cisplatin and carboplatin, have dominated the treatment of various cancers (Kostova, 2006). Platinum-based drugs are widely used in the treatment of cancer such as leukemia, lymphomas, melanoma, head-neck cancer, bladder cancer and gynecological tumors (Wong & Giandomenico, 1999). Cisplatin is one of the first platinum-based drugs discovered in the 1960s (Milacic, Fregona, & Dou, 2008).

2.4.8.1.Cisplatin

Cisplatin is a DNA-damaging agent that is widely used in cancer chemotherapy. It is one of the first platinum-based drugs discovered in the 1960s (D. Chen, Milacic, Frezza, & Dou, 2009). Cisplatin has significant activity in solid tumor malignancies, with successful therapeutic outcomes for head and neck, lung, ovarian, and testicular cancers (D. Chen, Milacic et al., 2009).

The traditionally accepted mechanism of action of cisplatin involves its cross-linking to DNA, forming intra- and inter- strand adducts, which unwind the duplex and

attract high-mobility-group domain and other proteins. The shielding effect of these proteins results in poor repair of the cisplatin-modified DNA, thereby leading to activation of several signaling transduction pathways (including those involving ATF, p53, p73, and MAPK) and ultimately cell apoptosis (Tanida, Mizoshita et al., 2012). However, our understanding of cisplatin-induced cell death remains limited, as it is a nonspecific drug that reacts not only with DNA, but also with proteins, resulting in several other proposed and studied mechanisms of action for cell death in addition to cell apoptosis (Gonzalez, Fuertes, Alonso, & Perez, 2001). Pestell et al. showed that populations of cisplatin treated cells were undergoing not only apoptosis, but also cell death via a necrotic route (Pestell, Hobbs, Titley, Kelland, & Walton, 2000).

Furthermore, the idea of apoptosis and necrosis as being two distinct mechanisms of cellular death has been challenged, and scientists have instead proposed a continuum of cellular death, where a cell fall on this continuum depends on specific factors such as the availability of energy and metabolic condition of the cell (Leist, Single, Castoldi, Kühnle, & Nicotera, 1997). Segal-Bendirdjian and Jacquemin-Sablon determined that cisplatin-induced cell death in L1210 leukemia cells was at least partly a result of an unfinished apoptotic program (Segal-Bendirdjian & Jacquemin-Sablon, 1995). In addition, Perez proposed that, cisplatin not only exerts DNA-damaging effects, but it damages molecules involved in cellular energy supply (i.e., ATP) and also destroys proteins involved in apoptosis (i.e., p53, Bax, Bcl-2, and caspases), leading to necrotic cell death (Perez, 1998).

Similar to other chemotherapeutic agents, the effect of cisplatin is commonly limited by the resistance of cancer cells. Cisplatin resistance can be intrinsic or acquired. Intrinsic resistance means that cancer cells retain certain featured gene

expression profiles contributing to resistance prior to cisplatin treatment. In contrast, acquired resistance occurs in cancer cells after cisplatin-induced epigenetic modulation and gene mutation (Leist, Single et al., 1997).

In clinical treatment, cisplatin often results in the development of chemoresistance, despite a consistent rate of initial responses. Acquired cisplatin resistance is also the most common cause of therapeutic failure and cancer recurrence.

In the context of leukemia, cisplatin, among other platinum-based agents, has been implicated as cytotoxic agent that has strong leukemogenic potential and puts patients at risk for developing therapy-related myeloid neoplasms (Ishikawa, Nakayama et al., 2014). In an analytical study of 18.657 testicular cancer patients by Travis et al., it was shown that a cumulative exposure of 650 mg cisplatin/m2 for testicular cancer treatment increased the relative risk of leukemia in these patients by 3.2-fold, while larger doses (1000 mg cisplatin/m2) were linked with six fold increase in relative risk (Travis, Andersson et al., 2000). Cisplatin's carcinogenic potential is thought to be augmented when combined with other carcinogenic therapies, such as radiation in the setting of concurrent therapy regimens (Dertinger, Avlasevich et al., 2014).

Cisplatin's role as a therapeutic agent for AML, on the other hand, is much less understood. Cisplatin has been previously considered as combination chemotherapy in relapsed or refractory AML. In a phase I trial by Seiter et al., five of 20 patients (15 of which had AML) demonstrated a significant reduction in bone marrow blasts, as cisplatin was thought to increase the sensitivity of leukemia cells to temozolomide by depleting MGMT (Seiter, Katragadda, Ponce, Rasul, & Ahmed, 2009). Similarly, Lee

et al. showed the combination of high dose cytarabine, etoposide, and cisplatin to be effective salvage chemotherapy in high-risk relapsed or refractory AML, with overall complete remission rate of 31% among 49 patients (Seiter, Katragadda et al., 2009).

2.4.9. Complications associated with common leukemia therapy

2.4.9.1.Cancer recurrence

Recurrence of cancer is when tumor comes back again commonly when the therapy is complete. Moreover, no signs or symptoms for the disease are seen during weeks, months or years. In some types of tumor, the recurrence is expected and considered as a part of the disease cycle for example CML (Kantarjian, O'Brien et al., 2002). Remission is a state in which no cancerous cells can be found in the body, can be either temporary or permanent. Sometimes remission is temporary, and patients' relapse, and cancer recurs in the same place where the disease first began (primary site) or in different places in the body (secondary site). Unfortunately, the most common causes of treatment failure and drug resistance are related to relapse where curing the cancer becomes more difficult (Giralt et al, 1994; Leukemia and Lymphoma Research, 2013). Moreover, patient's survival after relapse is poor and ranges from 21% to 33% (Rubintz et al', 2006). The rate of complete remission (CR) is related to age, for example patients with AML younger than 60 years have remission rates of 60-80% (Lowenberg et al, 1999), whereas remission rates of 40-65% was seen in those 60 years and older, who represent the majority of the AML population (Hiddemann et al, 1999; Leopold et al, 2002). A number of studies have shown that combination therapies between two chemotherapies agents induced much higher rates of CR, for instance, more than 40% of patients with AML achieved a CR and long-term survival following the treatment with cytosine arabinoside and an anthracyclin (Juliusson et al, 2005).

2.4.9.2.Resistance to chemotherapy

Development of multidrug resistance (MDR) against anti-cancer drugs is a very serious problem during the treatment of Leukemia and other cancers (Gottesman, 2002; Luqmani, 2008). Once MDR develops, using high doses of chemotherapeutic agents to overcome resistance is ineffective and my lead to further toxic effects and resistance are more stimulated (Ozben, 2006). Multidrug resistance severely limits effectiveness and inhibits cytotoxic effects of chemotherapy in a number of common cancers and is responsible for the overall poor efficacy of chemotherapy (Liscovitch et al, 2002; Akan et al, 2005). The resistance can be either acquired as a cellular response to drug exposure or inherited in some cancerous cells leading to altered target enzyme; increased drug degradation, decrease drug absorption and/ or enhanced DNA repair (Luqmani, 2008). Mutations within some vital genes for example p53 (tumor suppression gene) have been reported to play an important role in multidrug resistance via inhibiting apoptotic production within tumor cells (Gottesman et al,
2002).

2.4.9.3.Side effects

Cancer patients commonly experience side effects because of cancer treatments. Side effects following the anti-cancer agent treatment vary depending on several factors such as patient's age, health status, cancer type, size and how close the cancer is to other important organs ("Cancer diagnosis and treatment statistics," 2015). Nonetheless, there are a number of common side effects associated with the majority of chemotherapies, including; nausea, sickness, vomiting, feeling weak, tiredness, hair loss, depression and Low white blood cell count ("Cancer diagnosis and treatment statistics," 2015). In addition, high dose chemotherapy or radiotherapy is known to be

associated with a high risk of developing ovarian failure and infertility, this is mostly due to ovarian damage that often occurs after the treatment; however, it depends on patient's age and treatment protocol (Meirow & Nugent, 2001). Some side effects are serious medical conditions that need to be treated while; many side effects are inconvenient or upsetting but are not harmful to the patient's health and disappear when the treatment finished. Because blood cancer treatments have become more aggressive during the last 20 years (Redd, Montgomery, & DuHamel, 2001), the need for new treatments for Leukemia to improve patient's health and reduce the side effects associated with such therapy has become essential.

2.5.Ethnobotany and Medicine

2.5.1. Natural compounds overview

Ethnobotany and Ethnopharmacology are interdisciplinary fields of research that look specifically at the empirical knowledge of indigenous peoples concerning medicinal substances, their potential health benefits and their health risks associated with such remedies. Many of the plant-derived pharmaceuticals and phytomedicines currently in use were used by native people around the world. Some of this knowledge has been documented and codified or studied scientifically. The importance of ethnobotanical inquiry as a cost-effective means of locating new and useful tropical plant compounds cannot be over emphasized. Most of the secondary plant compounds employed in modern medicine were “first” discovered through ethnobotanical investigation. Analysis of the data on prescriptions dispensed from community pharmacies in the US from 1959 to 1980, indicates that 25% contained plant extracts or active principles derived from higher plants and at least 119 chemical substances, derived from 90 plant species, can be considered as important drugs currently in use in

one or more countries. Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine (Reddya et al., 2003).

2.5.2. Natural Sources as Potential Anti-Cancer Agents

The widespread nature of the use of medicinal plants and their contribution to human health, perhaps, is one of the most significant ways in which humans directly reap the benefits provided by biodiversity. For more than a decade, there has been considerable interest in the use of naturally occurring botanicals for prevention of various cancers (Aggarwal & Shishodia, 2006). Drug discovery from medicinal plants has played an important role in the treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer (Butler, 2004). Of all available anticancer drugs between 1940 and 2002, 40% were natural products or natural product-derived with another 8% considered natural product mimics (Newman et al., 2003).

Several epidemiological studies have shown that high intake of fruit and vegetables are associated with low incidence of a number of human cancers (Boffetta, Couto et al., 2010; Key, 2011; Neuhouser, Thompson, Coronado, & Solomon, 2004; Pavia, Pileggi, Nobile, & Angelillo, 2006). Fruits and vegetables are excellent source of fiber, vitamins, and minerals, but they also contain bioactive compounds, including polyphenols. Polyphenols are an integral part of human diet flavonoids and phenolic acids representing the majority of polyphenols present in fruits and vegetables such as pomegranate. These compounds have been shown to have anti-carcinogenic effects in vitro and in in vivo models by modulating important cellular and molecular mechanisms related to carcinogenesis such as modulation cell cycle and induction of

apoptosis (Brusselmans, De Schrijver, Heyns, Verhoeven, & Swinnen, 2003; Hafeez, Siddiqui et al., 2008; Zaini, Clench, & Le Maitre, 2011). Therefore, apoptotic induction and cell cycle arrest within tumor cells has become excellent targets for potential cancer treatments and are proposed to decrease mortality from malignancy (Dorai & Aggarwal, 2004; Paschka, Butler, & Young, 1998).

2.5.3. Mechanisms by Which Natural Sources Exert Anti-Cancer Effects

2.5.3.1.Targeting cell cycle

Disruption of the normal regulation of cell cycle progression and division are important events in the development of cancer. In the last few decades, with advancements in understanding of the mechanisms of oncogenesis, apoptosis induction, along with the improved understanding on the effects of chemotherapy on healthy and cancerous cells, researchers have gained greater understanding of the critical role that cell cycle regulation plays in malignant transformation and in the development of resistance to chemotherapy. It is now increasingly apparent that the cell cycle plays a critical role in the development of resistance to chemotherapy. These observations have led to the development of a new anticancer therapeutics in clinical development today for improving the efficacy of targeted therapeutics and overcoming resistance to anticancer drugs; specifically, the use natural compounds as anti-cancer agents (Bailon-Moscoso, Romero-Benavides, & Ostrosky-Wegman, 2014; Cragg & Newman, 2013; Farnsworth, Akerele, Bingel, Soejarto, & Guo, 1985; Mann, 2002; Newman & Cragg, 2007). A number of natural compounds that inhibit the cell cycle arrest have proven effective for killing cancer cells in vitro, in vivo and in clinical settings. In fact, more than 60% of currently used anticancer agents are derived from natural sources supporting the notion that natural compounds are high-impact sources of new “lead

compounds" or new potential therapeutic agents (Cragg & Newman, 2013; Newman & Cragg, 2007).

2.5.3.2.Targeting apoptosis

Apoptosis helps to establish a natural balance between the generation of new cells (cell division) and loss of cells (cell death) by destroying excess, damaged, or abnormal cells. However, the balance between survival and apoptosis often tips towards the former in cancer cells. Dis-regulation in pro-apoptotic or anti-apoptotic proteins can inhibit the apoptotic process and allow cells to proliferate. Leukemia cells could cause this imbalance and evade apoptosis though numerous mechanisms (Hanahan & Weinberg, 2011; Lessene, Czabotar, & Colman, 2008) (see section 2.2.1). Various natural products and their bioactive compounds have been found to induce apoptosis through both intrinsic and extrinsic pathways (Vermeulen, Van Bockstaele et al., 2003).

2.6.Guggulsterone

2.6.1. Chemical structure

Guggulsterone [4, 17(20)-pregnadiene-3, 16-dione] is a plant polyphenol and an active component of gugulipid extracted from the gum resin of the guggul tree; *Commiphora mukul*. Guggulsterone exists as either of two forms; *E*-guggulsterone and *Z*-guggulsterone (Figure 2).

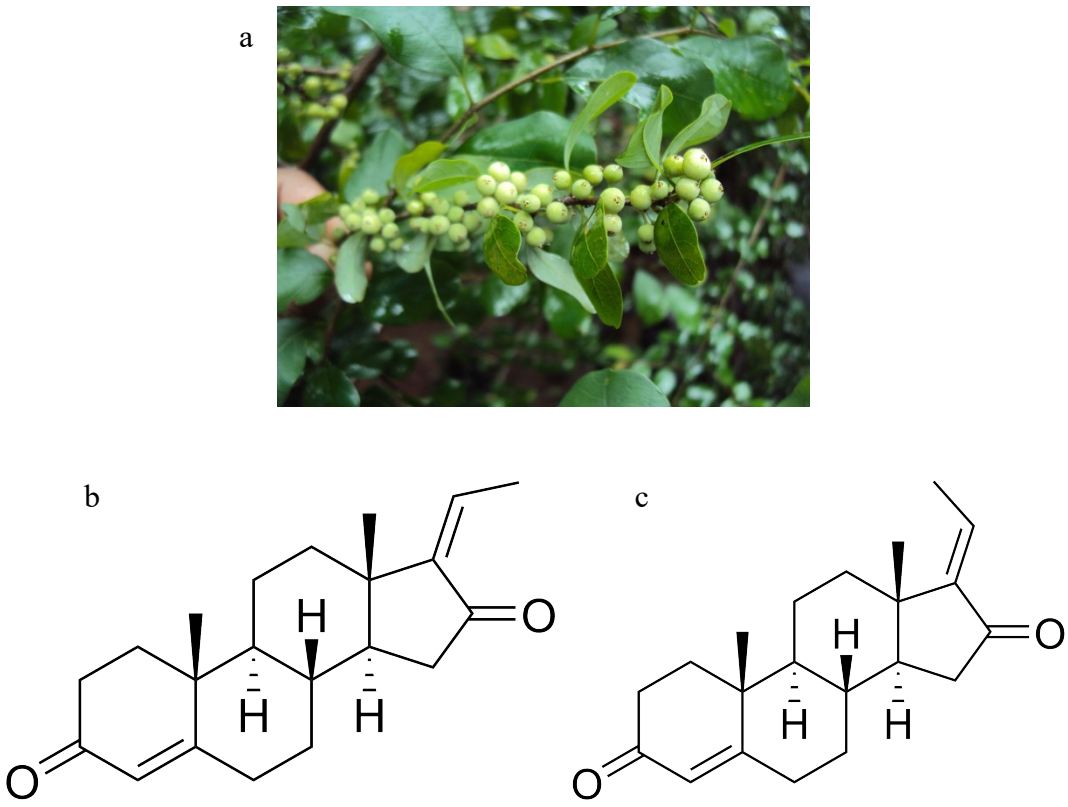

Figure 6. (a) The Plant *Commiphora mukul*. The chemical structure of Guggulsterone isoforms, Z -Guggulsterone (b) and E-Guggulsterone (c)

2.6.2. Treatment implications of Guggulsterone

Guggulsterone (GS) has been broadly used for centuries to treat multiple human diseases and disorders, including obesity, arthritis, and hyperlipidemia.(Satyavati, Dwarakanath, & Tripathi, 1969; Sinal & Gonzalez, 2002; Urizar, Liverman et al., 2002). Moreover, there accumulating evidence about the key role of GS in cholesterol homeostasis regulation via increasing the transcription of bile salt export

pump (Cui, Huang et al., 2003; Deng, Yang, Radke, Yang, & Yan, 2007; Owsley & Chiang, 2003). Furthermore, GS has been shown to play an important role in nutritional metabolism as it has been found to inhibit cholesterol synthesis in the liver via antagonism of the FXR and the bile-acid receptor (Szapary, Wolfe et al., 2003). Besides, GS has been widely used for years for treating of hyperlipidemia (Dev, 1999; Satyavati, 1988). Several studies have demonstrated that GS decreases low density lipoprotein cholesterol and triglyceride levels in serum and increases high density lipoprotein cholesterol levels (Nityanand, Srivastava, & Asthana, 1989; R. B. Singh, Niaz, & Ghosh, 1994). Specifically, E and Z isoforms of GS have been recognized as active ingredients for lipid-lowering (Beg, Singhal, & Afzaal, 1996). Interestingly, it has been demonstrated that the lipid lowering effect of GS in liver are due to inhibition of bile acid farnesoid X receptor (FXR) as confirmed from FXR knockout mice studies (Urizar, Liverman et al., 2002) via acting as an antagonist of FXR (Cui, Huang et al., 2003; Owsley & Chiang, 2003; Wu, Xia et al., 2002), thus preventing the expression of FXR agonist-mediated genes (Urizar, Liverman et al., 2002; Urizar & Moore, 2003).

2.6.3. Anti-cancer activity of Guggulsterone and mechanisms of action

In the past few decades, research has revealed that the active components of GS possess cancer chemopreventive and therapeutic potential to prevent and treat cancers. A number of studies have shown that GS induce apoptotic cell death in various cancer types, including pancreatic, colon, esophageal, breast, and prostate cancers (D. W. Ahn, Seo et al., 2012; An, Cheon et al., 2009; Guan, Hoque, & Xu, 2014; Samudio, Konopleva, Safe, McQueen, & Andreeff, 2005; Shishodia, Sethi, Ahn, & Aggarwal, 2007; S. V. Singh, S. Choi et al., 2007; Xiao & Singh, 2008),

via activation of caspases, increased expression of genes of Bcl-2 family members, and generation of reactive oxygen intermediates. Furthermore, GS has been shown to strongly inhibit the activation of various survival signaling pathways, including, PI3-kinase/AKT, JAK/STAT and nuclear factor-kB (NF-kB) in various cancer cells (Cheon, Kim et al., 2006; Ichikawa & Aggarwal, 2006; Shishodia & Aggarwal, 2004), that are involved in the regulation of growth and inflammatory responses via regulation of antiapoptotic and inflammatory genes. In addition, GS has also been shown to suppress the ionizing radiation (IR)-mediated activation of NF-κB and augments the radio sensitivity of human cancer cell lines (Choudhuri, Degraff, Gamson, Mitchell, & Cook, 2011). In addition, GS is known to be an antagonist of FXR, a bile acid receptor which was found to cause anticancer activity by contributing to the regulation of apoptosis (K. S. Ahn, Sethi et al., 2008; De Gottardi, Dumonceau et al., 2006; Deng, Yang et al., 2007; Guan, Li, Yang, Hoque, & Xu, 2013; Kapoor, 2008; Peng, Raufman, & Xie, 2012). Furthermore, GS is reported to reduce cell growth as well as prevents IR-induced DNA damage repair (Choudhuri, Degraff et al., 2011) and GS has been shown to induce apoptosis in a wide range of cancer cells (Jiang, Xiao et al., 2013; R. J. Leeman-Neill, S. E. Wheeler et al., 2009; Macha, Rachagani et al., 2013; Shishodia, Sethi et al., 2007; S. V. Singh, S. Choi et al., 2007; S. V. Singh, Zeng et al., 2005; Xiao & Singh, 2008).

2.7.Drug combinations

For many years, drug combinations have been used for treating different diseases. During the past century, attempts have been made to quantitatively measure the dose-effect relationships of each drug alone and its combinations and to determine whether

or not a given drug combination would produce a synergistic effect. These applications are most noticeable in the areas of anti-cancer drug research. In drug combinations, different drugs may target on different targets, or different cell subpopulations simultaneously. Drugs with different mechanisms could also be combined to enhance the effect of single drugs and to treat cancer cells more effectively.

There are several benefits for drug combination regimens. First of all, the efficacy of the therapeutic effect could be increased in combinations. Moreover, the dosage of each drug could be decreased to reduce adverse effects, while increasing or at least maintaining the same efficacy. Furthermore, selective synergism or efficacy synergism could be provided against target during drug combination. Lastly, the development of drug resistance in patients could be minimized. For these therapeutic benefits, drug combinations have been widely used and became the leading choice for treating cancers.

3. MATERIALS

3.1.Cell culture

3.1.1. Cell lines

3.1.1.1.K562s

Human chronic myeloid leukemia (K562s) cells (CRL-3343) were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA.

3.1.1.2.U937

Human monoblastic leukemia (U937) cells (CRL-1593.2) were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA.

3.1.1.3.THP1

Human acute monocytic leukemia (THP1) cells (TIB-202) were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA.

3.1.2. Media and supplements

RPMI-1640 medium, fetal bovine serum, and Penicillin-streptomycin were obtained from Invitrogen (Paisley, UK).

3.2. Drugs

(Z)-Guggulsterone (GS) and cisplatin were purchased from Tocris Bioscience (Bristol, UK).

3.3. Other chemicals

Antibodies against caspase-9, caspase-8, Bid, Bcl-xL, phospho AKT and cleaved caspase-3, caspase-3 were purchased from Cell Signaling Technologies (Beverly, MA). Cytochrome c, PARP and GAPDH antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). XIAP antibody was purchased from Abcam (Cambridge, England). Annexin V-FITC, propidium iodide staining solution,

Hoechst 33342 Solution, BD Cytofix/Cytoperm plus fixation and permeabilization solution kit, and BD MitoScreen (JC-1) Kit were purchased from BD Biosciences (NJ, United States). CCK-8 kit and N-acetyl cysteine (NAC) was obtained from Sigma-Aldrich (St. Louis, MO, United States). z-VAD-FMK was purchased from Calbiochem (San Diego, CA, United States). CellROX Green, MitoSOX Red, and ThiolTracker Violet were purchased from Invitrogen (MA, United States).

3.4.Apparatus and instrumentation

Tecan Spark multimode microplate reader (Tecan, BioTek Instruments Inc. Winooski, VT, USA). Cell culture was conducted under sterile conditions in a class-ll type A/B3 Biosafety cabinet (NuAire Inc., Plymouth, MN, USA), and all cultures were maintained in a CO_2 incubator containing a HEPA filter (Thermo Forma Scientific, Inc, Marietta, OH, USA). Centrifugation was conducted using a low speed Jouan CR3-I centrifuge (Jouan by Thermo Electron Corporation, Waltham, MA, USA) and a MC2 DESAGA centrifuge (Sarstedt-Gruppe, Montreal, Quebec). Other general laboratory equipment included the following: Adventurer balance (OHAUS, Ontario, Canada), Vortex Jr. Mixer from Scientific Industries Inc, PSU-20i multi-functional orbital shaker (Biosan, Riga, Latvia), SUB Aqua Pro Water Bath (Grant Instruments^{TM}), freezer vials (VWR), and Eppendorf pipettes (Fisher Scientific) were used.

4. METHODS

4.1.Preparation of guggulsterone

Guggulsterone was dissolved in dimethylsulfoxide (DMSO) as a 15 mM stock solution and stored at -20°C for the in vitro experiments. Further dilution was done in cell culture medium as required.

4.2.Preparation of cisplatin

Cisplatin was dissolved in dimethylsulfoxide (DMSO) as a 3 mM stock solution and stored at -20°C for the in vitro experiments. Further dilution was done in cell culture medium as required.

4.3.Cell culture

4.3.1. In vitro propagation of cell lines

K562s leukemia cell line were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

K562-r leukemia cell line were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin, and 1mM imatinib at 37°C in a humidified atmosphere containing 5% CO2.

U937 leukemia cell line were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

4.3.2. Sub-culturing of cells

Cells were sub-cultured every 2 to 3 days by removing the culture medium by aspiration. Cultures were maintained at 1 x 10^6 cells/mL by the addition of appropriate complete media, cells were aspirated, and appropriate aliquots of the cell suspension were added to new culture flasks.

4.4.Treatment of cells with drugs:

4.4.1. Treatment with guggulsterone

Cells were grown to at least 90% confluence and treated with varying doses of Guggulsterone (12.5, 25, 50) μM by adding it directly to cells in fresh complete medium for varying time periods.

4.4.2. Treatment with cisplatin

Cells were grown to at least 90% confluence and treated with varying doses of cisplatin (5, 10) μM by adding it directly to cells in fresh complete medium for varying time periods.

4.4.3. Treatment with Guggulsterone in combination with Cisplatin

K562s, U937, THP1 cell lines were seeded at 75×10^4 cells/mL in T-25 cell culture flasks. Then, cells were treated with the lowest effective doses of guggulsterone (12.5, 25) μM and cisplatin (5, 10) μM by adding the drugs directly to cells fresh complete medium and incubated for for varying time periods with the treatment.

4.5.Cell viability assays

4.5.1. Trypan blue exclusion cell viability assay

To determine the number of cells and their viability, Trypan blue dye (Cat. # 1450021; BioRad, Hercules, CA, USA) exclusion tests were carried out. In brief, 10 µL of cells re-suspended in fresh media were mixed with 10 µL of 0.4% solution of trypan blue dye (Sigma-Aldrich) for 1 min. Cells were immediately counted using TC20 automated cell counter (Bio-Rad, Hercules, CA, USA). All counts were performed in three technical duplicates of each sample. Means were calculated for each subculture.

4.5.2. CCK-8 cell viability assay

K562s, U937 and THP1 cells were treated with GS (5, 10, 25, 50 μM) and evaluated for cell viability using CCK-8 colorimetric method. Briefly, treated K562s, K562-r and U937 cells were seeded into 96-well plates (1.0 x 10^4 cells/well) in a total volume of 100 μL media and maintained at 37°C in a humidified atmosphere of 5% CO2 for 24, 48, 72, 96 h. Following incubation, 10 μL CCK-8 solution was added to each well. The cells were then incubated at 37°C in a humidified atmosphere containing 5% CO2 for 2 h prior to measuring the optical density (OD) at 450 nm using Microplate Reader (Tecan, BioTek Instruments Inc. Winooski, VT, USA). Six replicate measurements with three independent experiments were conducted. Percentage of cell viability was calculated as OD of the experiment samples/OD of the control × 100%.

4.6.Annexin-V binding apoptosis assay

Annexin-V binding measures another characteristic feature of apoptosis: phosphatidyl serine flipping across the plasma membrane. This assay was used to confirm that the cancerous cells treated with GS were indeed undergoing apoptosis. Briefly, K562s, U937 and THP1 cells were treated with various doses of GS (5 μM, 12.5 μM, 25 μM, 50 μM) and incubated for 48 h. Cells were washed with PBS and stained with fluorescein-conjugated annexin-V and propidium iodide in 1× annexin binding buffer for 20 min. Flow cytometry was used to quantify cells that were either viable or had undergone apoptosis or necrosis after treatment (Badmus, Ekpo, Hussein, Meyer, & Hiss, 2015). Percentage apoptosis was expressed as a combination of cells present in early and late apoptosis (Prabhu, Siveen et al., 2017).

4.7.DNA laddering

The DNA laddering is an important feature of the apoptotic cells to observe the cellular apoptosis as well as nucleotide cleavage. Briefly, 2 x 10^6 of K562s, U937, and THP1 cells were treated with the indicated doses of guggulsterone alone/or in combination with cisplatin for 48 h, cells were harvested, and DNA was isolated using DNA laddering kit from Roche as described previously (34). After measuring the DNA, 2 lg of DNA was run on 1% agarose gel containing 0.1% ethidium bromide. After 2 h of running at 75 v, the gel was visualized under UV light using gel documentation system (Proteinsimple, Alphaimager Mini, USA).

4.8.Cell lysis and immunoblotting

Treated K562S, U937 and THP1 cells were centrifuged, and the cell pellets were washed in ice-cold PBS. Then, cell lysates were prepared using 2X Laemmli buffer containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris-HCl. After words, the mixture was boiled at 95–100°C for 5 min. Quantification of proteins were performed using the ND-1000 (Nanodrop Technologies, Thermoscientific, USA). Then, the reducing agent β-mercaptoethanol was added to the cell lysates, and 25–50 μg of protein samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Billerica, MA). The residual binding sites on the filters were blocked by incubating with TBST (10 mM Tris, pH 8.0, 150 mM sodium chloride, 0.05% Tween 20), 20% bovine serum albumin for 1–3 h at room temperature or overnight at 4 °C. The filters were subsequently incubated with the indicated antibodies. Then, the blots were developed and visualized under a chemidoc system (Amersham, Bio-Rad, USA).

4.9.H2AX, active caspase-3 and cleaved PARP quantification

H2AX, active caspase-3 and cleaved PARP were quantified by flow cytometry.

After treatment with guggulsterone alone, cisplatin alone, and in combination, K562s, U937, and THP1 cells were fixed and permeabilized using BD Cytofix/ Cytoperm Plus Fixation and Permeabilization Solution Kit, as per protocol from the manufacturer. 0.5 × 106 cells in Stain Buffer (FBS) were stained with 3 µL each of H2AX (pS139)-Alexa Fluor 647, Rabbit Anti- Active Caspase-3- BV605 and PARP Cleaved Form- AF700 antibodies for 30 minutes at room temperature. The cells were washed with Stain Buffer (FBS) and then analyzed by flow cytometry.

4.10. Cell cycle analysis by flow cytometry

Considering that both guggulsterone and cisplatin affected the cell viability inducing death in the three cell lines, their effects on cell cycle distribution after treatment with the indicated doses were analyzed. Briefly, following the treatment with the indicated doses, cells were harvested and lysed as described in (Hussain, Al-Rasheed et al., 2006). Briefly, 0.5×10^6 cells were stained with Hoechst 33342 solution (10 µg/mL) and then analyzed by flow cytometry BD LSRFortessa analyzer (BD Biosciences, NJ, United States) (Siveen, Mustafa et al., 2014).

4.11. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential of leukemia cells was measured using 5,5',6,6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1, Sigma, St. Louis, MO). JC-1 is a positively charged fluorescent compound which is taken up by mitochondria proportionally to the inner mitochondrial membrane potential (Smiley, Reers et al. 1991). When a critical concentration is exceeded, JC-1 monomer forms J-aggregates and becomes fluorescent red, altering the fluorescence properties of the compound. In apoptotic cells, the mitochondrial membrane potential collapses, and the JC-1 cannot accumulate within the mitochondria and remains in the cytoplasm in a

green fluorescent monomeric form. Accordingly, the ratio of red (J-aggregate) to green (monomeric JC-1) emission is directly proportional to the mitochondrial membrane potential. Briefly, K562s, U937, and THP1 cells were treated with guggusterone alone, cisplatin alone, and in combination. Then, cells were incubated for 48 h at 37°C. Cells were then washed with warmed serum-free medium. Afterwards, 0.5×10^6 cells were and stained with 10 μM JC-1 for 15 min at 37ºC as per instructions from the kit manufacturer. The cells were then washed twice with 1× assay buffer and finally analyzed by flow cytometry.

4.12. Reactive oxygen species (ROS) quantification

CellROX Deep Red Oxidative Stress Reagent is a fluorogenic probe designed to reliably measure reactive oxygen species (ROS) in live cells. The signals from CellROX Deep Red Reagent is localized in the cytoplasm. The production of superoxide by mitochondria was quantitated using the MitoSOX Red reagent. It is rapidly oxidized by superoxide but not by other reactive oxygen species and reactive nitrogen species. Cells were treated with guggulsterone (0, 1, 2.5, 5 μM) for 24 h and finally analyzed by flow cytometry for quantification of ROS and superoxide.

4.13. Calculation of combination index (CI)

The specific interaction between guggulsterone and cisplatin on K562s, U937, and THP1 leukemia cell lines was evaluated by the CI analysis. Drug combination synergy was performed using CompuSyn software (Chou & Martin, 2005). CI values and CI-Fa plot (plot representing CI versus Fa, the fraction affected by a particular dose) were calculated by CompuSyn program (Compusyn Inc., Paramus, NJ, USA). All experiments were repeated at least three times.

4.14. Data collection and statistical analysis

Absorbance measurements were collected using Microplate Reader (Tecan, BioTek Instruments Inc. Winooski, VT, USA). The significance of differences between different treatment groups was determined by one-way ANOVA using GraphPad Prism v7.0 (GraphPad Software Inc, California, USA). Values of $P < 0.05$ were considered statistically significant. $*$ denotes $p < 0.05$. In all figures, data is expressed as the mean ± standard deviation (S.D), with the vertical error bars denoting the S.D.

5. RESULTS

5.1.Anti-tumor activity of guggulsterone alone

5.1.1. Anti-proliferative activity of guggulsterone in leukemia cells

To determine the effect of guggulsterone on the viability of leukemia cells. K562s, U937, and THP1 leukemia cells were treated with GS for various time periods as previously described and cell viability was measured using CCK-8 coloremetric viability assay. The results of the cytotoxicity of guggulsterone has been described in Figures 7-10 and LC50 values were calculated using GraphPad Prism. Table 3 gives a summary of IC50 values of the guggulsterone in K562s, U937, and THP1 leukemia cell lines. Figure 11 gives a graphical representation of the IC50 values obtained. The results showed that guggulsterone is toxic to K562s, THP1, and U937 cells at incubation periods of 48 hours and above, with IC50 values < 100 μM (table 3, Figure 14).

5.1.1.1.Determination of the minimum effective guggulsterone concentration

In order to calculate the idea concentration for GS treatment, the cytotoxic effect of guggulsterone was firstly determined in K562s cell lines with doses ranging from 2.5 μM to 100 μM for 72 h treatment. CCK-8 coloremetric viability assay demonstrated that guggulsterone decreased the numbers of viable cells in a dose-dependent manner (Figure 7). Guggulsterone at doses lower than 12.5 μM showed non-significant reduction in cell viability. Doses of 12.5 μM and above showed significant reduction in cell viability % ($P<0.05$). Although, 100 μM (the highest concentration employed) showed significant reduction in cell viability %, it was observed that guggulsterone

precipitates at high dose. Thus, from these results, the concentration of guggusterone to be used for further experiments was fixed at 12.5 – 50 µM.

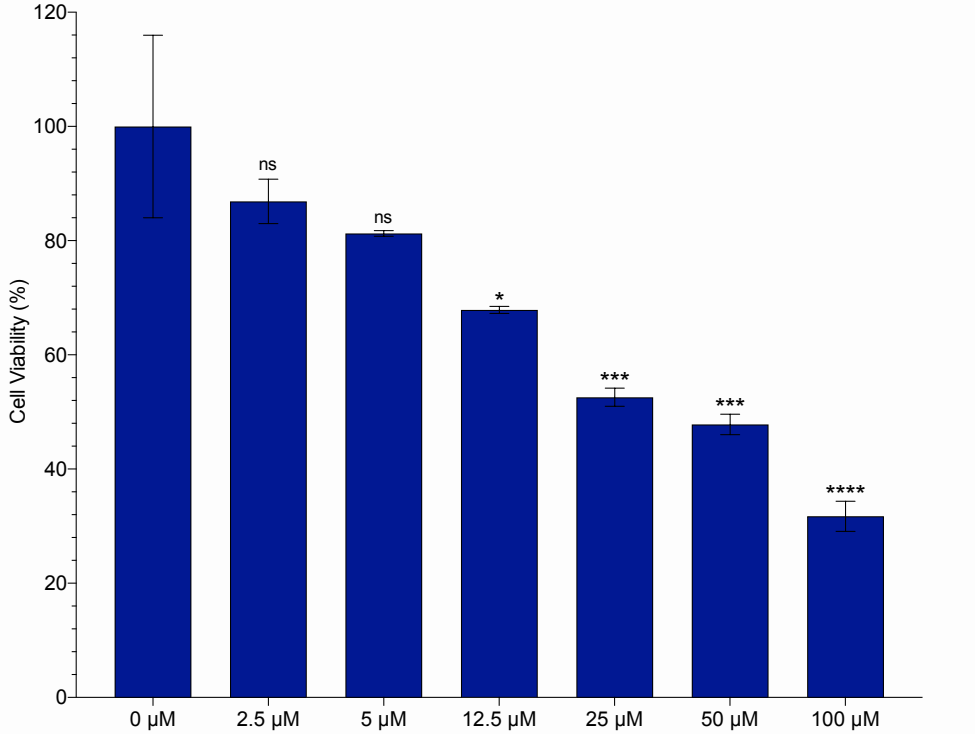

Figure 7. Effect of guggulsterone (GS) on K562s cell viability after 72 hours using CCK-8 coloremetric viability assay. Each value is expressed as mean ± SEM of three independent experiments, with six technical replicates each. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.1.2. Dose- and time-dependent anti-proliferative activity of guggulsterone in K562s leukemia cells.

To assess the effect of guggulsterone on cell viability in K562s cell lines, cells were treated with increasing concentrations (5, 12.5, 25, and 50 and 50 µm) of guggulsterone for 24, 48, 72, and 96 hrs. A time- and dose-dependent decrease in cell viability was observed in K562s cell lines (Figure 8).

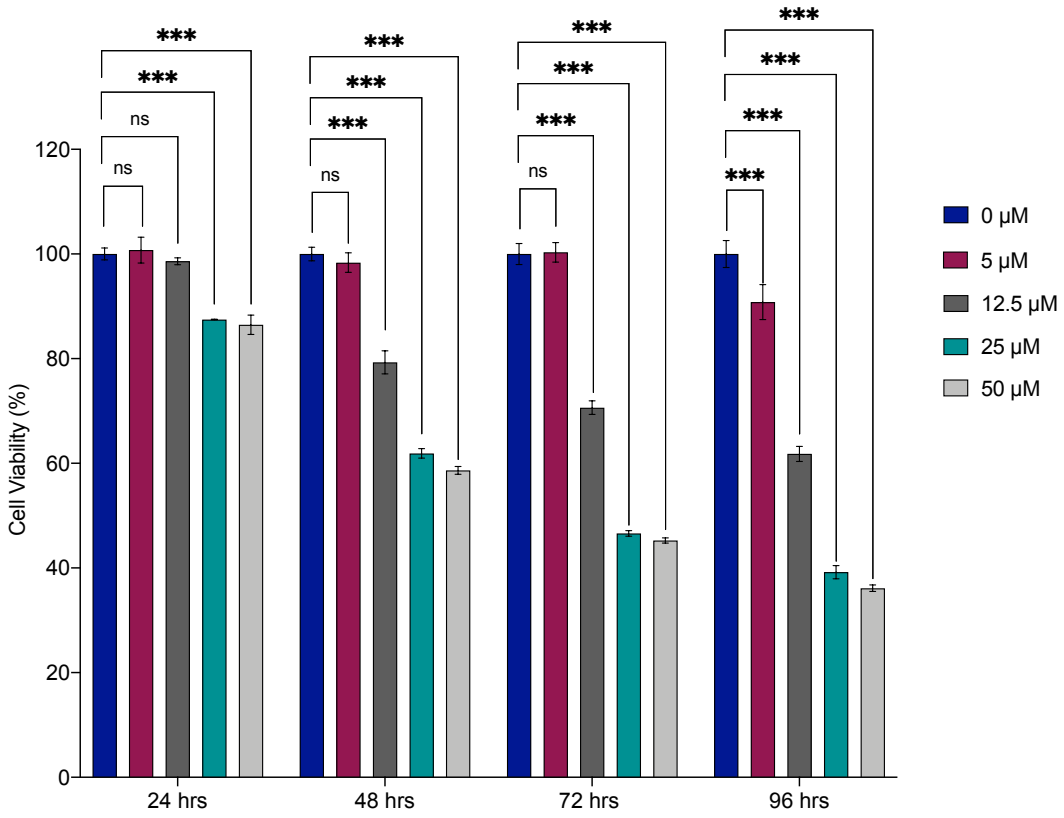

Figure 8. Effect of guggulsterone (GS) on K562s cell viability after 24, 48, 72, and 96 hours. Inhibitory effects of guggulsterone on the viabilities of THP1 cells was evaluated by CCK-8 viability assay. Each value is expressed as mean ± SEM of three independent experiments, with six technical replicates each. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.1.3. Dose- and time-dependent anti-proliferative activity of guggulsterone in U937 leukemia cells.

To assess the effect of guggulsterone on cell viability in U937 cell lines, cells were treated with increasing concentrations (5, 12.5, 25, and 50 and 50 µm) of guggulsterone for 24, 48, 72, and 96 hrs. A time- and dose-dependent decrease in cell viability was observed in U937 cell lines (Figure 9).

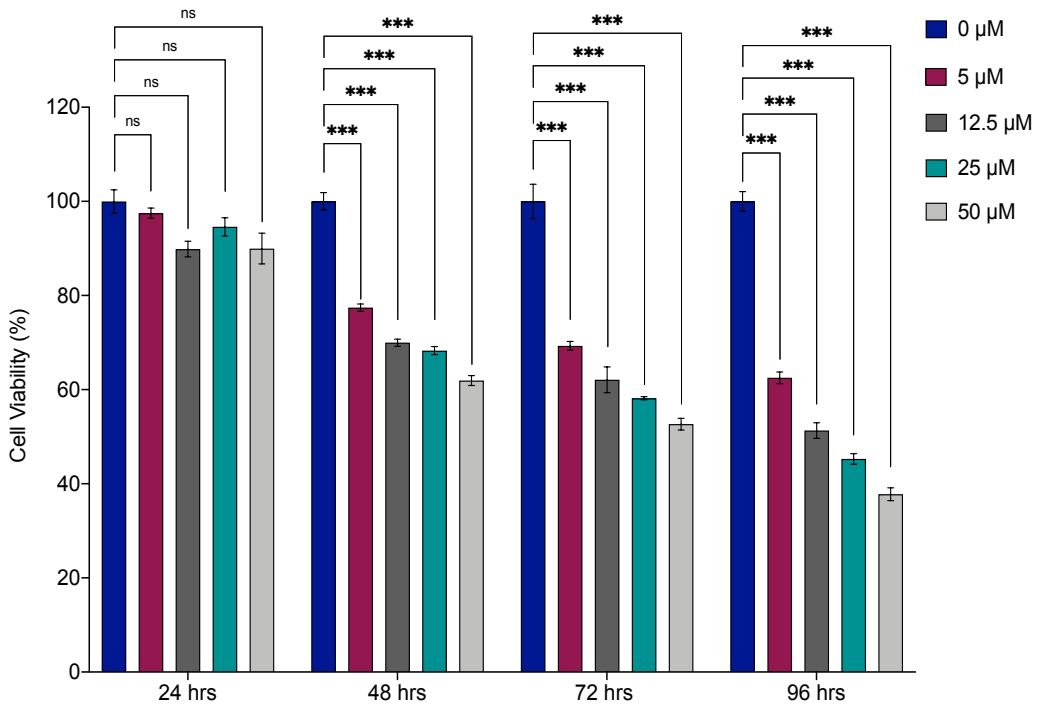

Figure 9. Effect of guggulsterone (GS) on U937 cell viability after 24, 48, 72, and 96 hours. Inhibitory effects of guggulsterone on the viabilities of THP1 cells was evaluated by CCK-8 viability assay. Each value is expressed as mean ± SEM of three independent experiments, with six technical replicates each. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.1.4. Dose- and time-dependent anti-proliferative activity of guggulsterone in THP1 leukemia cells.

To assess the effect of guggulsterone on cell viability in THP1 cell lines, cells were treated with increasing concentrations (5, 12.5, 25, and 50 and 50 µm) of guggulsterone for 24, 48, 72, and 96 hrs. A time- and dose-dependent decrease in cell viability was observed in THP1 cell lines (Figure 10).

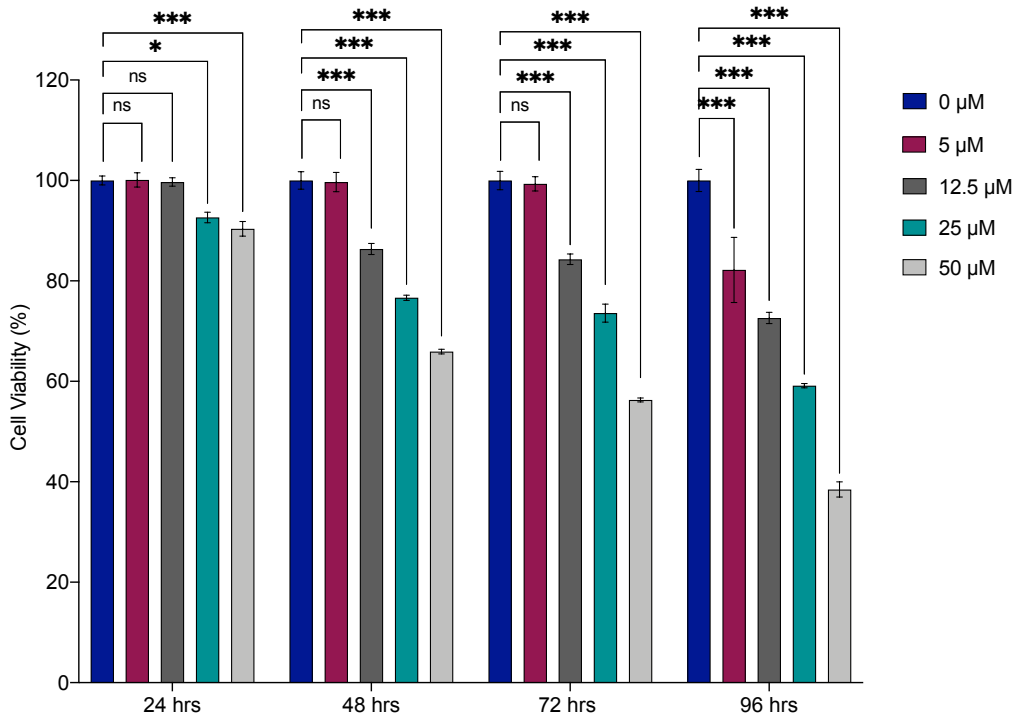

Figure 10. Effect of guggulsterone (GS) on THP1 cell viability after 24, 48, 72, and 96 hours. Inhibitory effects of guggulsterone on the viabilities of THP1 cells was evaluated by CCK-8 viability assay. Each value is expressed as mean ± SEM of three independent experiments, with six technical replicates each. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.1.5. Summary of LC50 values

Table 3. List of the LC50 values of guggulsterone in K562s, THP1, and U937 leukemia cell lines at four different time points.

Cell lines	Duration (hours)	LC_{50}[a] (μM)
K562s	24	236.5±25.3
	48	61.6±7.2
	72	36.1±3.8
	96	23.1±2.9
THP1	24	454.4±68.6
	48	92.4±5.8
	72	69.1±5.0
	96	32.2±2.8
U937	24	374.7±86.3
	48	52.8±8.9
	72	30.5±5.4
	96	16.4±1.7

LC50, 50% of lethal concentration;
LC50 were calculated as mean ± SEM (n=3);
"a" the LC50 value was determined by sigmoidal curve fitting.

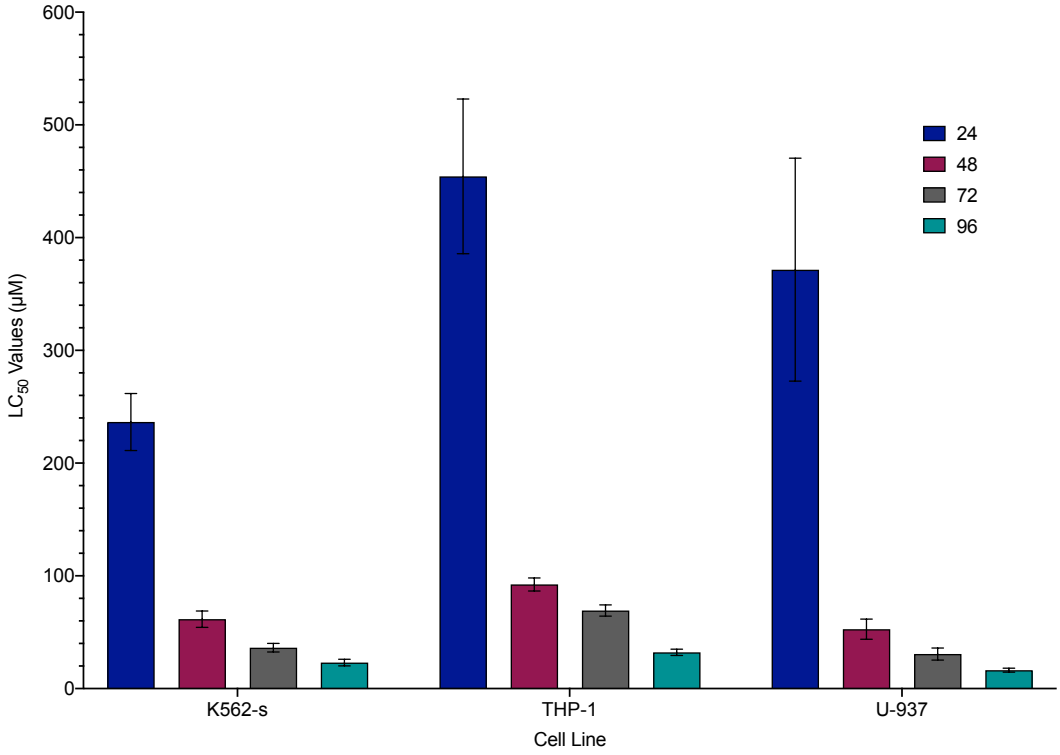

Figure 11. LC50 values of guggulsterone towards leukemia cell lines. The LC50 values of guggulsterone (table 3) are represented as a bar graph. The error bars represent the standard error of the mean (SEM).

5.1.2. Effects of guggulsterone on apoptosis in leukemia cells

To determine if guggulsterone inhibits cellular viability by induction of apoptosis, K562s, U937, and THP1 cells were treated with various doses of guggulsterone, as indicated, Annexin V-FITC staining and propidium iodide accumulation was used to differentiate early apoptotic cells (Annexin V-FITC^{+} and PI^{-}) from living cells (Annexin V-FITC^{-} and PI^{-}). A significant ($P < 0.05$) decrease in the number of live cells and increase in the number of early apoptotic cells when assessed at 48 h post treatment

treatment with guggulsterone was observed in acute leukemia cells (U937 and THP1) but not chronic leukemia cells (K562s) (Figures 12-14). In addiition, within the acute leukemia cell lines different levels of sensitivity were observed, U937 was more affected compared to THP1 leukemia cells (Figure 12).

To confirm apoptosis induction, we analyzed DNA fragmentation, which is another hallmark of apoptosis. K562s, U937, and THP1 cells were treated with various doses of guggulsterone as indicated and DNA was isolated using an apoptotic DNA-laddering kit from Roche. As shown in Figure 12-14, guggulsterone caused fragmentation of DNA, a characteristic of apoptotic cell death.

5.1.2.1.Effects of guggulsterone on apoptosis in K562s cells

Guggulsterone-induced apoptosis was evaluated by annexin V and PI staining in THP1 cells. The mean percentages of total apoptosis are plotted in the bar graph in Figure 12. As shown, guggulsterone significantly induces apoptosis in THP1 leukemia cell line ($P<0.05$). Following 48h treatment with 12.5µM, 25µM, and 50µM of GS. As shown in the figure, there is a trend of increase in % of total apoptosis in a dose-dependent manner after incubation with increasing doses of GS for 48 h. In the cells treated with GS, the % cells with total apoptosis was increased significantly from 19.8% in the control group to 22.4% and 23.2%, following treatment with 50, and 100 µM GS. However, no significant apoptosis was observed at GS doses less than 25µM.

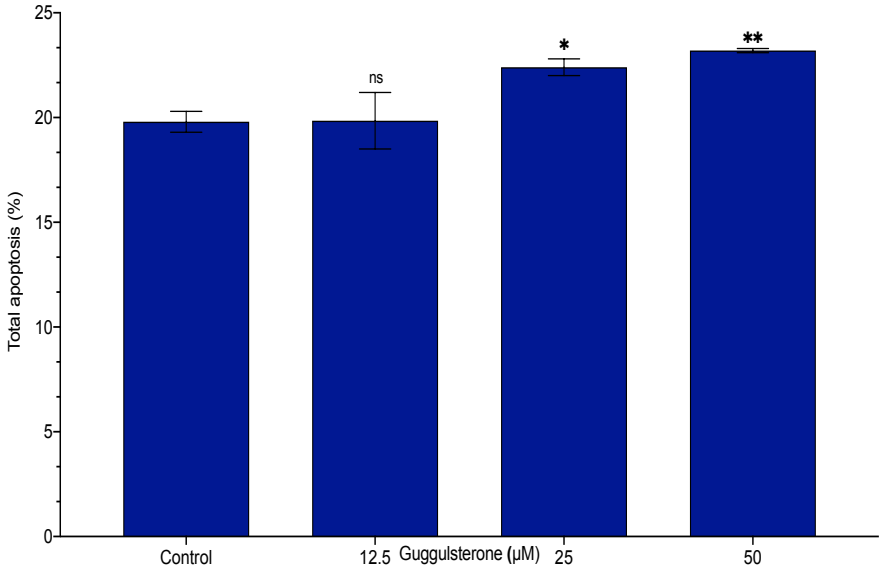

Figure 12. The effects of guggulsterone on K562s cell growth and apoptosis. Histogram representation of the quantitative percentage of total apoptosis of THP1 cells. The cells were treated with guggulsterone (12.5, 25, and 50 µM) for 48 h, labelled with FITC annexin V and PI, and analyzed by flow cytometry. Each value is expressed as mean ± SD of three independent samples. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.2.2.Effects of guggulsterone on apoptosis in U937 cells

Guggulsterone-induced apoptosis was evaluated by annexin V and PI staining in THP1 cells. The mean percentages of total apoptosis are plotted in the bar graph in Figure 13. As shown, guggulsterone significantly induces apoptosis in THP1 leukemia cell line ($P<0.05$). Following 48h treatment with 12.5µM, 25µM, 50, and 100µM of GS. As shown in the figure, there is a trend of increase in % of total apoptosis in a dose-dependent manner after incubation with increasing doses of GS for 48 h. In the cells treated with GS, the % cells with total apoptosis was increased significantly from 12.4% in the control group to 22.2% and 39.2%, following treatment with 50, and 100 µM GS. However, no significant apoptosis was observed at GS doses less than 50µM.

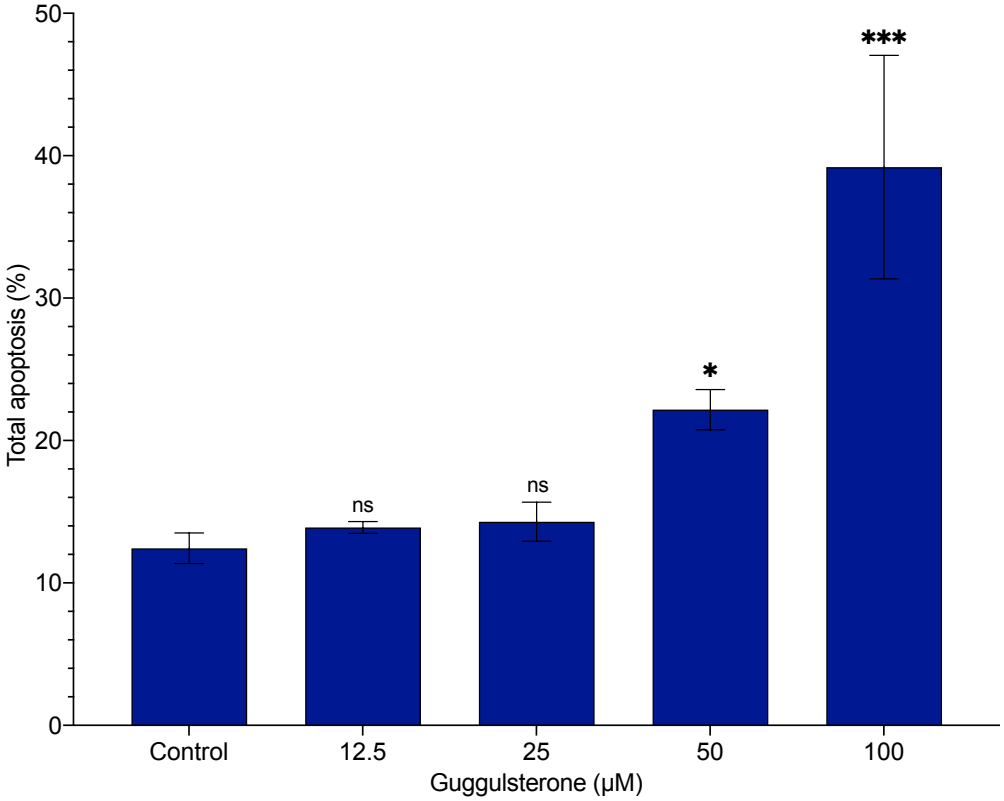

Figure 13. The effects of guggulsterone on U937 cell growth and apoptosis. Histogram representation of the quantitative percentage of total apoptosis of U937 cells. The cells were treated with different concentrations of GS (12.5 µM, 25 µM, 50 µM, and 100 µM) for 48 h, labelled with FITC annexin V and PI, and analyzed by flow cytometry. Each value is expressed as mean ± SD of three independent samples. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.2.3. Effects of guggulsterone on apoptosis in THP1 cells

Guggulsterone-induced apoptosis was evaluated by annexin V and PI staining in U937 cells. The mean percentages of total apoptosis are plotted in the bar graph in Figure 13. As shown, guggulsterone significantly induces apoptosis in U937 leukemia cell line ($P<0.05$). Following 48h treatment with 12.5μM, 25μM, 50, and 100μM of GS. As shown in the figure, there is a trend of increase in % of total apoptosis in a dose-dependent manner after incubation with increasing doses of GS for 48 h. In the cells treated with GS, the % cells with total apoptosis was increased significantly from 11.4% in the control group to 15.72%, 17.28%, and 22.15% following treatment with 25, 50, and 100 μM GS. However, no significant apoptosis was observed at GS doses less than 25μM.

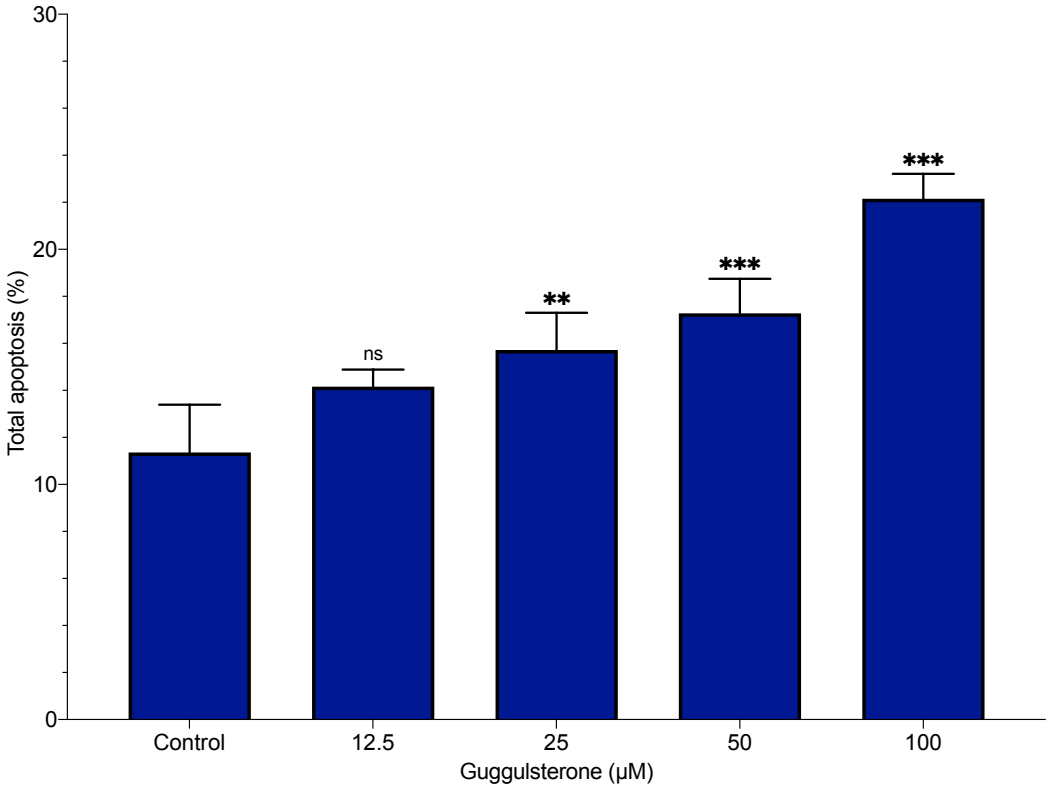

Figure 14. The effects of guggulsterone on THP1 cell growth and apoptosis. Histogram representation of the quantitative percentage of total apoptosis of THP1 cells. The cells were treated with different concentrations of GS (12.5 µM, 25 µM, 50 µM, and 100 µM) for 48 h, labelled with FITC annexin V and PI, and analyzed by flow cytometry. Each value is expressed as mean ± SD of three independent samples. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.3. Guggulsterone-induced apoptosis: effect on PARP and caspases

5.1.3.1. Guggulsterone induces the activation of caspases and enhances PARP cleavage in K562s cell lines.

We investigated whether caspases were activated in K562s leukemia cells under guggulsterone treatment, and whether guggulsterone-mediated apoptosis involves activation of caspase-8 in mitochondrial or intrinsic apoptotic pathway. K562s cell lines were treated with guggulsterone and immunoblotted with antibodies against caspase-3; caspase-9; PARP, and HSP-60. As shown in Figure 15, guggulsterone induced the activation of caspase-3 and induced the cleavage of PARP in a dose-dependent manner, indicating the execution of intrinsic apoptosis in leukemia cells (Figure 15).

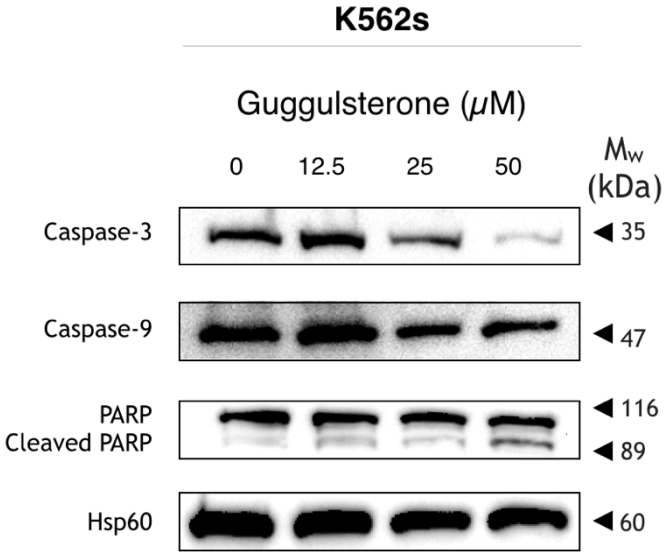

Figure 15. Guggulsterone-induced activation of caspase-3, caspase-9, and cleavage of PARP and caspase-8 in K562s cell lines. Cells were treated with increasing doses of guggulsterone (12.5, 25, 50 μM) for 48 hrs. Cells were lysed and immunoblotted with antibodies against caspase-3; caspase-9; PARP, and HSP-60. Gugglsterone–induced activation of caspases 3, caspase-9 and cleavage of PARP in K562s leukemia cells when treated for 48 hrs. A representative of three independent experiments is depicted in the figure.

5.1.3.2. Guggulsterone induces the activation of caspases and enhances PARP cleavage in U937 cell lines.

We investigated whether caspases were activated in U937 leukemia cells under guggulsterone treatment, and whether guggulsterone-mediated apoptosis involves activation of caspase-9 in intrinsic mitochondrial apoptotic pathway. U937 cell lines were treated with guggulsterone and immunoblotted with antibodies against caspase-3, caspase-9, and HSP-60. As shown in Figure 16, guggulsterone induced the activation of caspase-3 in a dose-dependent manner, indicating the execution of intrinsic apoptosis in leukemia cells.

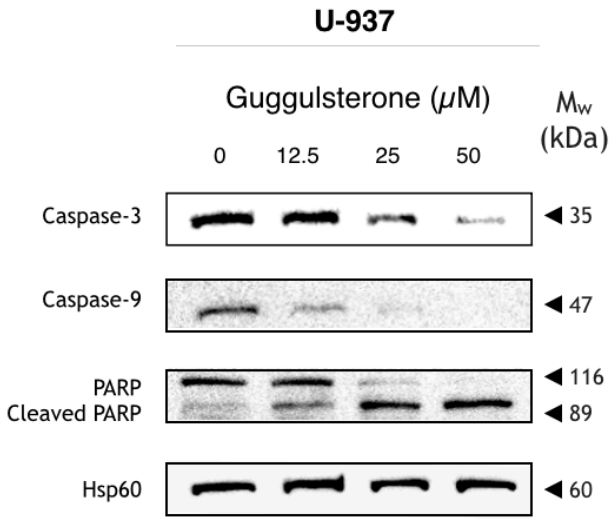

Figure 16. Guggulsterone-induced activation of caspase-3 in U937 cell lines. Cells were treated with increasing doses of guggulsterone (12.5, 25, 50 μM) for 48 hrs. Cells were lysed and immunoblotted with antibodies against caspase-3, caspase-9, and HSP-60. Gugglsterone–induced activation of caspase-3 in U937 leukemia cells when treated for 48 hrs. A representative of three independent experiments is depicted in the figure.

5.1.3.3. Guggulsterone induced the activation of caspases and enhances PARP cleavage in THP1 cell lines.

We investigated whether caspases were activated in THP1 leukemia cells under guggulsterone treatment**,** and whether guggulsterone-mediated apoptosis involves activation of caspase-8 in mitochondrial or intrinsic apoptotic pathway. THP1 cell lines were treated with guggulsterone and immunoblotted with antibodies against cleaved caspase-3, and HSP-60. As shown in Figure 10, guggulsterone induced the cleavage of cleaved caspase-3 in a dose-dependent manner, indicating the execution of intrinsic apoptosis in leukemia cells.

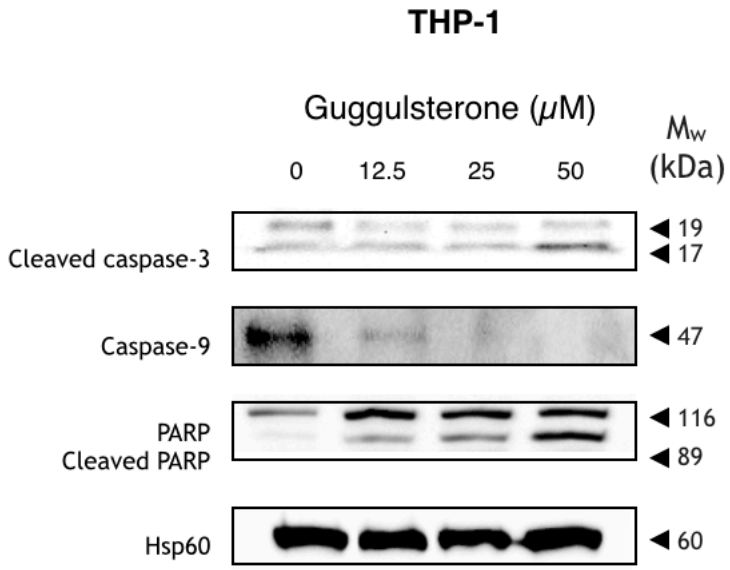

Figure 17. Guggulsterone-induced cleavage of caspase-3 in THP1 cell lines. Cells were treated with increasing doses of guggulsterone (12.5, 25, 50 μM) for 48 hrs. Cells were lysed and immunoblotted with antibodies against cleaved caspase-3 and HSP-60. Gugglsterone–induced cleavage of caspase-3 in THP1 leukemia cells when treated for 48 hrs. A representative of three independent experiments is depicted in the figure.

5.1.4. Guggulsterone regulates the expression of pro-apoptotic and anti-apoptotic proteins in leukemia cells

For cell culture convenience, we used THP1 cells as an example to further elaborate the anticancer mechanisms of GS in leukemia. THP1 cell lines were treated with increasing doses of GS [12.5, 25, 50 μM] for 48 h, lysed and immunoblotted with antibodies against Bax, Bcl-2, Bid, Bcl-xL, and HSP-60. As shown in Figure 18, GS increased the expression of pro-apoptotic proteins; Bax and Bid, and decreased the expression of anti-apoptotic proteins; Bcl-xl and Bcl-2 in THP1 cells.

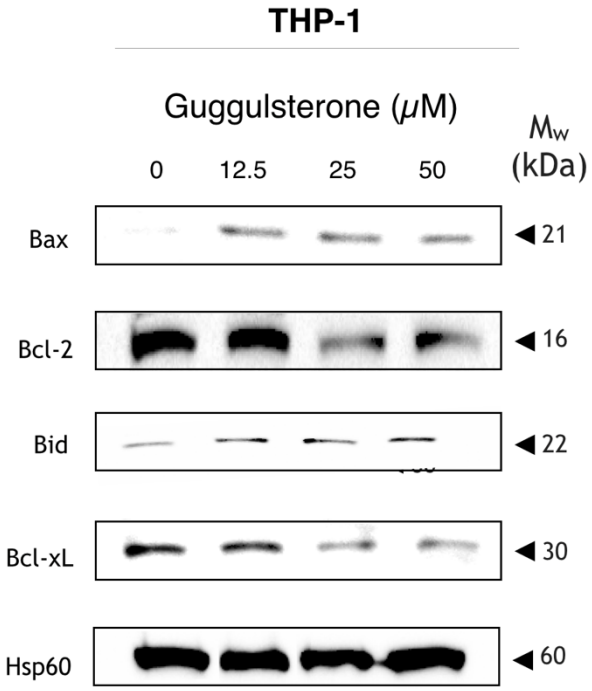

Figure 18. Guggulsterone increased the expression of pro-apoptotic proteins and decreased the expression of anti-apoptotic proteins in THP1 cell lines. Cells were treated with increasing doses of guggulsterone (12.5, 25, 50 μM) for 48 hrs. Cells were lysed and immunoblotted with antibodies against Bax, Bid, Bcl-xl, Bcl-2 and HSP-60. Gugglsterone increased the expression of pro-apoptotic proteins; Bax and Bid, and decreased the expression of anti-apoptotic proteins; Bcl-xl and Bcl-2 in THP1 cell lines in THP1 leukemia cells when treated for 48 hrs. A representative of three independent experiments is depicted in the figure.

5.1.5. Guggulsterone down-regulates intracellular apoptosis inhibitor proteins (IAP) in leukemia cells

THP1 cell lines were treated with increasing doses of GS [12.5, 25, 50 μM] for 48 h, lysed and immunoblotted with antibodies against xIAP, cIAP-1, cIAP-2, and HSP-60. As depicted in Figure 19, treatment with GS for 48 h suppressed the expression of MMP-2 and MMP-9 in THP1 cells, compared with in the control group.

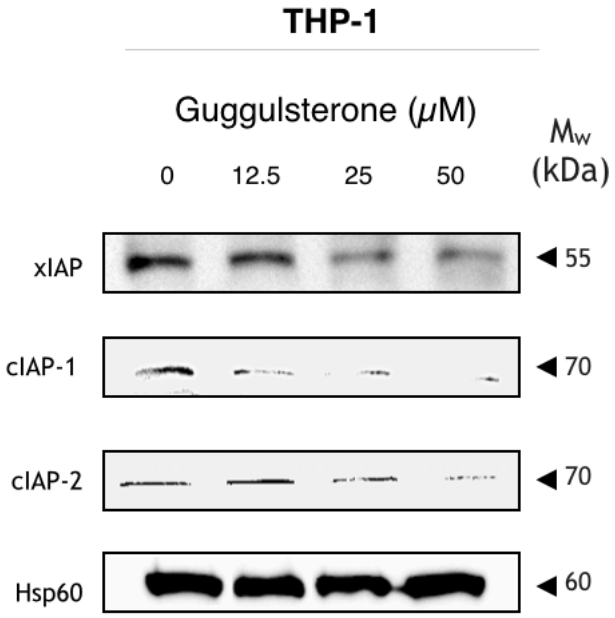

Figure 19. Guggulsterone decreased the expression of IAPs in THP1 cell lines. Cells were treated with increasing doses of guggulsterone (12.5, 25, 50 μM) for 48 hrs. Cells were lysed and immunoblotted with antibodies against xIAP, cIAP-1, cIAP-2 and HSP-60. Gugglsterone decreased the expression of xIAP, cIAP-1, cIAP-2 in a dose-dependent manner in THP1 cells when treated for 48 hrs. A representative of three independent experiments is depicted in the figure.

5.1.6. Effects of guggulsterone on cell cycle distribution in leukemia cells

To further investigate the molecular mechanism(s) involved in the observed growth inhibition, the cell cycles of K562s, U937, and THP1 cells exposed to GS were examined. Cells were treated with varying concentrations (12.5, 25, 50 μM) for 48 h and cell cycle analysis was performed using flow cytometry. As depicted in Figures 20-21, differential effects on cell cycle stage following GS treatment were observed dependent on the cell type investigated and dose of GS treatments.

5.1.6.1.Effects of guggulsterone on cell cycle distribution in K562s cells

The cell cycle phase distribution of K562s cells treated with GS at 48 hours is depicted in Figure 20. As shown in the figure, the pattern of distribution at different phases for K562s was altered in a dose-dependent manner after incubation with increasing doses of GS for 48 h. Following GS treatment, significant reduction of K562s cells at G0/G1 was found at doses 25 μM and 50 μM, with concomitant significant increases in sub G0/G1 phase at doses 50 μM. As summarized in Figure 20, the G0/G1 cells decreased from 56.45% in the control group to 54.85%, 51.1%, and 50.85% respectively following 48 h-treatment with 12.5 μM, 25 μM, and 50 μM of GS, whereas cells at sub G0/G1 phase increased from 5.4% in the control group to 6.25%, 6.55%, and 9.5% respectively following treatment with 12.5 μM, 25 μM, and 50 μM of GS. However, no apparent signs of cell cycle arrest and only small but statistically insignificant variation were observed in all other phases of the cell cycles, suggesting cell death mechanisms were involved in the anti-tumorigenic action of GS, especially around the dose of LC50 (about 50 μM).

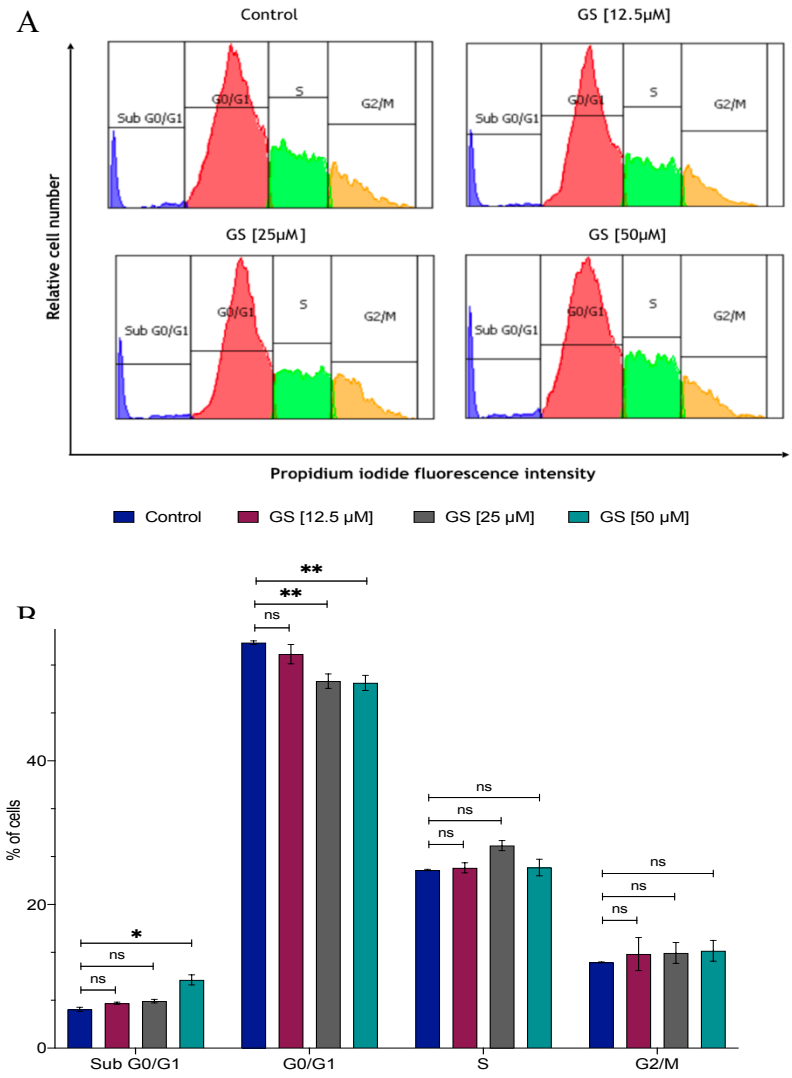

Figure 20. Effects of guggulsterone on cell cycle distribution of K562s cells. (A) Representative graphs obtained by flow cytometric analysis representing relative cell number at the different phases following treatment with 12.5, 25, and 50 μM of GS. (B) Representative histograph of the dose-dependent effect of GS on the cell cycle distribution of K562s cells. Cell population percentages of sub-G0/G1, G0/G1, S and G2/M phases are indicated in the figure. Statistical analyses are shown as averages with indicated standard errors (n = 3). GS increases the sub-G0/G1 phase in K562s cells. The values represent the mean ± SEM from three independent samples. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.6.2. Effects of guggulsterone on cell cycle distribution in U937 cells

The cell cycle phase distribution of U937 cells treated with GS at 48 hours is depicted in Figure 21. As shown in the figure, the pattern of distribution at different phases for U937 was significantly altered in a dose-dependent manner after incubation with increasing doses of GS for 48 h. Following GS treatment, s significant reduction of U937 cells at G0/G1 was found at dose 100 μM, with concomitant significant increase in sub G0/G1 phase at dose 100 μM. As summarized in Figure 20, the G0/G1 cells decreased from 57.45% in the control group to 42.85% following 48 h-treatment with 100 μM of GS, whereas cells at sub G0/G1 phase increased from 22.9% in the control group to 47.45% following treatment with 100 μM of GS. However, doses below 100 μM did not cause any change in % of cells in any of the phases. Furthermore, no apparent signs of cell cycle arrest and only small but statistically insignificant variation were observed in all other phases of the cell cycles, suggesting cell death mechanisms were involved in the anti-tumorigenic action of GS, especially around the dose of LC50 (about 100 μM).

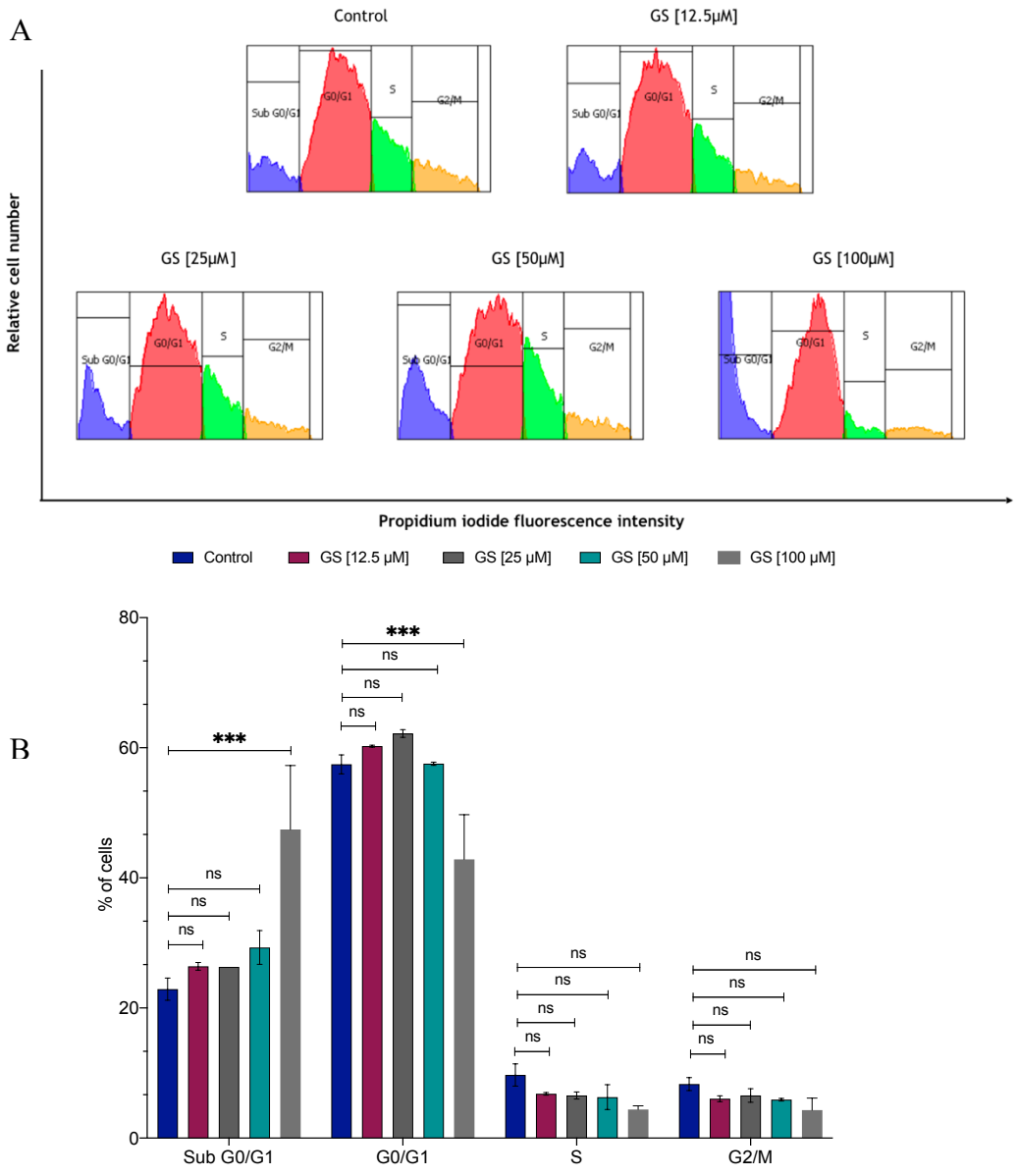

Figure 21. Effects of guggulsterone on cell cycle distribution of U937 cells. (A) Representative graphs obtained by flow cytometric analysis representing relative cell number at the different phases following treatment with 12.5, 25, 50, and 100 μM of GS. (B) Representative histograph of the dose-dependent effect of GS on the cell cycle distribution of U937 cells. Cell population percentages of sub-G0/G1, G0/G1, S and G2/M phases are indicated in the figure. Statistical analyses are shown as averages with indicated standard errors (n = 3). GS increases the sub-G0/G1 phase in U937 cells. The values represent the mean ± SEM from three independent samples. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.6.3. Effects of guggulsterone on cell cycle distribution in THP1 cells

The cell cycle phase distribution of THP1 cells treated with GS at 48 hours is depicted in Figure 22. As shown in the figure, the pattern of distribution at different phases for THP1 was significantly altered in a dose-dependent manner after incubation with increasing doses of GS for 48 h. Following GS treatment, s significant reduction of THP1 cells at G0/G1 was found at doses 12.5 μM, 25 μM, and 50 μM, with concomitant significant increases in sub G0/G1 phase at doses 25 μM, and 50 μM. As summarized in Figure 20, the G0/G1 cells decreased from 43.15% in the control group to 36.85%, 28.4%, and 24.45% respectively following 48 h-treatment with 12.5 μM, 25 μM, and 50 μM of GS, whereas cells at sub G0/G1 phase increased from 17.2% in the control group to 22.85%, 37.65%, and 41.95% respectively following treatment with 12.5 μM, 25 μM, and 50 μM of GS. However, no apparent signs of cell cycle arrest and only small but statistically insignificant variation were observed in all other phases of the cell cycles, suggesting cell death mechanisms were involved in the anti-tumorigenic action of GS, especially around the dose of LC50 (about 50 μM).

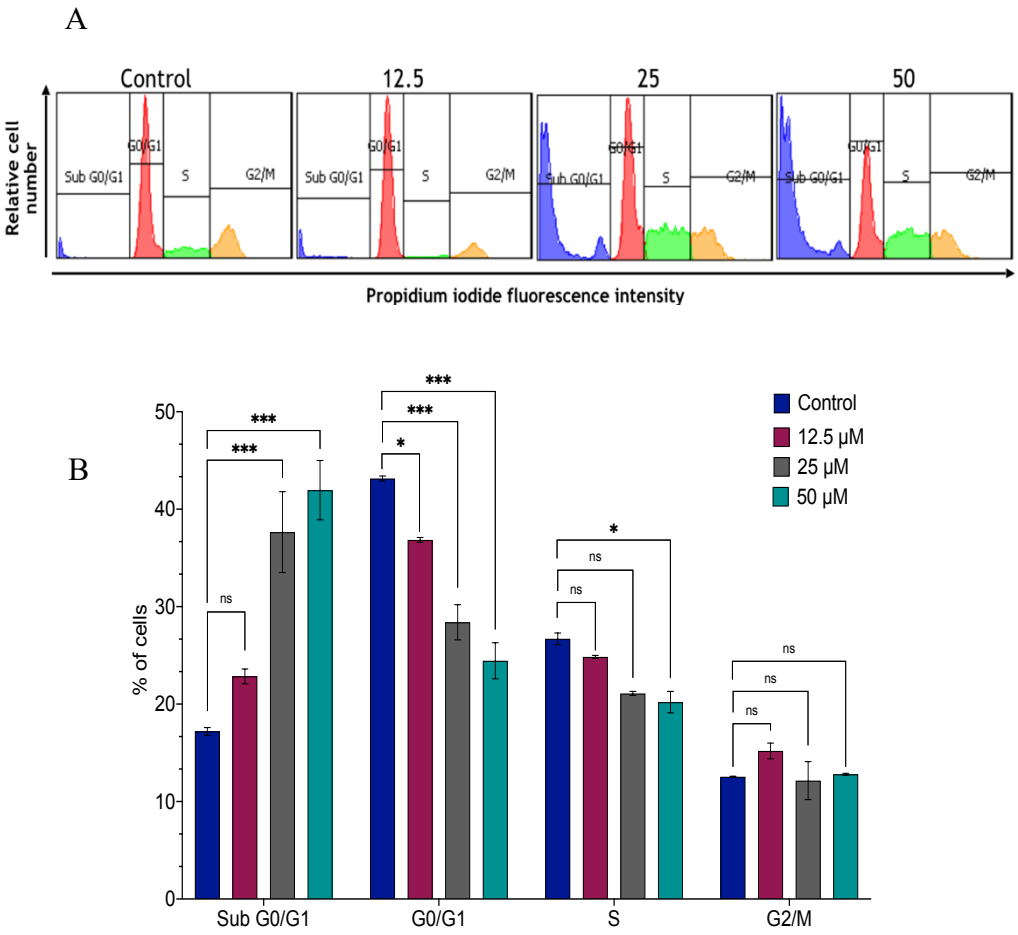

Figure 22. Effects of guggulsterone on cell cycle distribution of THP1 cells. (A) Representative graphs obtained by flow cytometric analysis representing relative cell number at the different phases following treatment with 12.5, 25, and 50 µM of GS. (B) Representative histograph of the dose-dependent effect of GS on the cell cycle distribution of THP1 cells. Cell population percentages of sub-G0/G1, G0/G1, S and G2/M phases are indicated in the figure. Statistical analyses are shown as averages with indicated standard errors (n = 3). GS increases the sub-G0/G1 phase in THP1 cells. The values represent the mean ± SEM from three independent samples. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.7. Effects of guggulsterone on the expression of cell cycle regulators in leukemia cell lines

To further determine the underlying mechanisms of regulating cell cycle, THP1 cells were used to examine the expression of cyclin A2. Cyclin A2 levels was suppressed in a dose-dependent manner in THP1 cells (Figure 23).

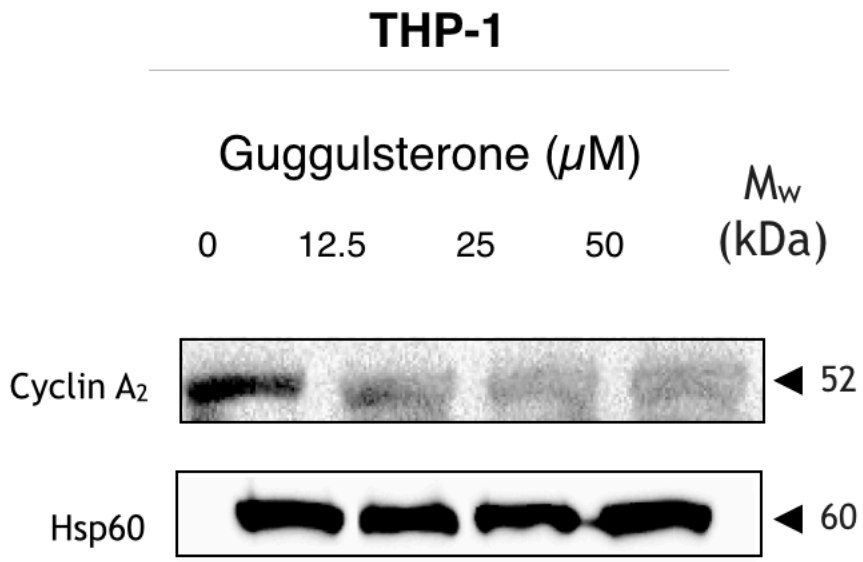

Figure 23. Guggulsterone effect on CyclinA2 in THP1 cell lines. Cells were treated with increasing doses of guggulsterone (12.5, 25, 50 μM) for 48 hrs. Cells were lysed and immunoblotted with antibodies against cyclin A2 and HSP-60. A representative of three independent experiments is depicted in the figure.

5.1.8. Effects of guggulsterone on mitochondrial membrane potential (MMP) in leukemia cells

The collapse of the mitochondrial membrane potential is a common event in the apoptotic pathway that leads to mitochondrial dysfunction and production of ROS. To better understand the mechanism of GS anti-proliferative effects on acute leukemia cells, MitoPotential Dye staining was carried out as described in Materials and methods. Loss of mitochondrial membrane potential was measured in U937, and THP1 cells by flow cytometry. Data shown in Figures 24-25.

5.1.8.1.Effect of guggulsterone on MMP in human U937 leukemia cell lines

The results in Figure 24 showed that treatment with GS reduced the MMP in U937 cells. As monitored by JC-1 staining, there is a trend of increase in % loss of MMP in a dose-dependent manner after incubation with increasing doses of GS for 48 h. In the cells treated with GS, the % cells with loss of MMP was increased significantly from 19.95% in the control group to 45.55% following treatment with 100μM GS. However, no significant loss of MMP was observed at GS doses less than 100μM.

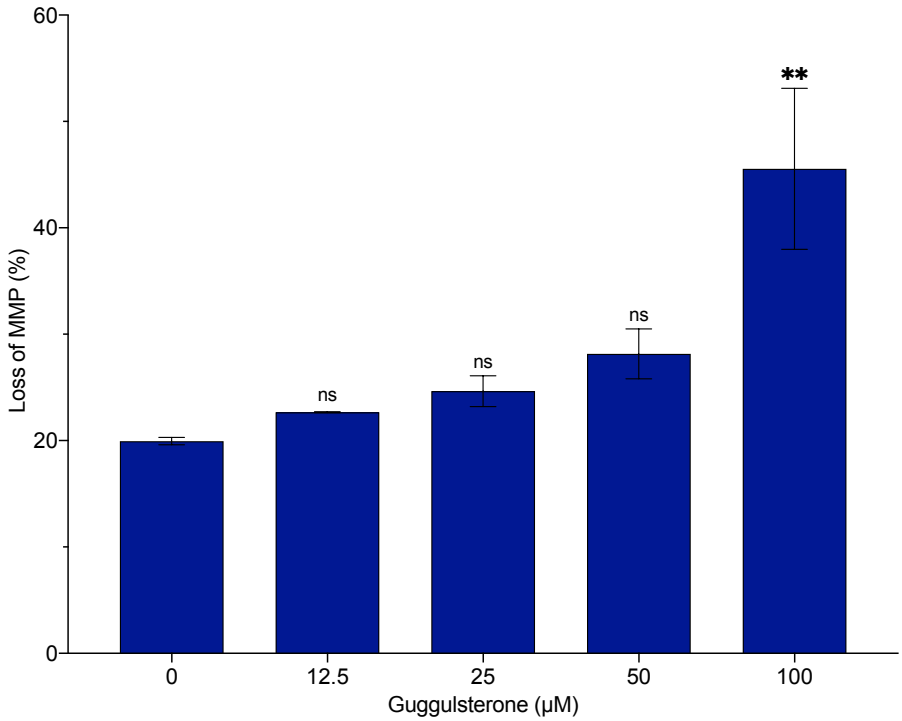

Figure 24. Guggulsterone induces loss of mitochondrial membrane potential (MMP) in U937 cells. U937 cells were treated with 0,12.5, 25, 50, and 100 μM GS for 48 h. The mitochondrial membrane potential (MMP) was measured by flow cytometry. The values represent the mean ± SD from three independent samples. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.8.2.Effect of guggulsterone on MMP in human THP1 leukemia cell lines

The results in Figure 25 showed that treatment with GS reduced the MMP in THP1 cells. As monitored by JC-1 staining, there is a trend of increase in % loss of MMP in a dose-dependent manner after incubation with increasing doses of GS for 48 h. In the cells treated with GS, the % cells with loss of MMP was increased significantly from 24.2% in the control group to 44.9% and 49.8% following treatment with 25 and 50µM GS respectively. However, no significant loss of MMP was observed at GS doses less than 25µM.

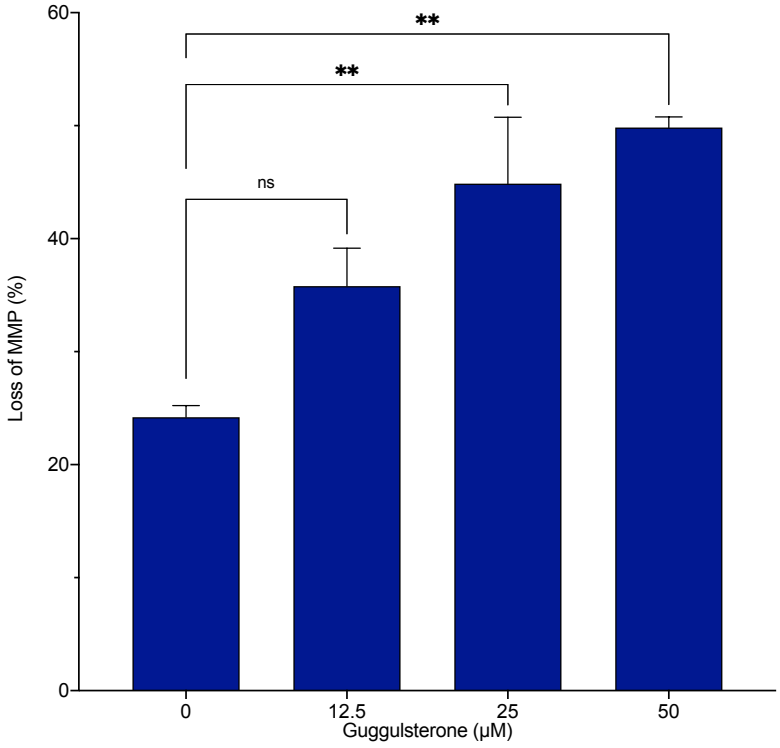

Figure 25. Guggulsterone induces loss of mitochondrial membrane potential (MMP) in THP1 cells. THP1 cells were treated with 0,12.5, 25, and 50 μM GS for 48 h. The mitochondrial membrane potential (MMP) was measured by flow cytometry. The values represent the mean ± SD from three independent samples. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.1.9. *Guggulsterone regulates the expression of mitochondrial integrity proteins in* leukemia *cells*

THP1 cell lines were treated with increasing doses of GS [12.5, 25, 50 μM] for 48 h, lysed and immunoblotted with antibodies against MMP-2, MMP-9, and HSP-60. As depicted in Figure 26, treatment with GS for 48 h suppressed the expression of MMP-2 and MMP-9 in THP1 cells in a dose-dependent manner, compared with in the control group.

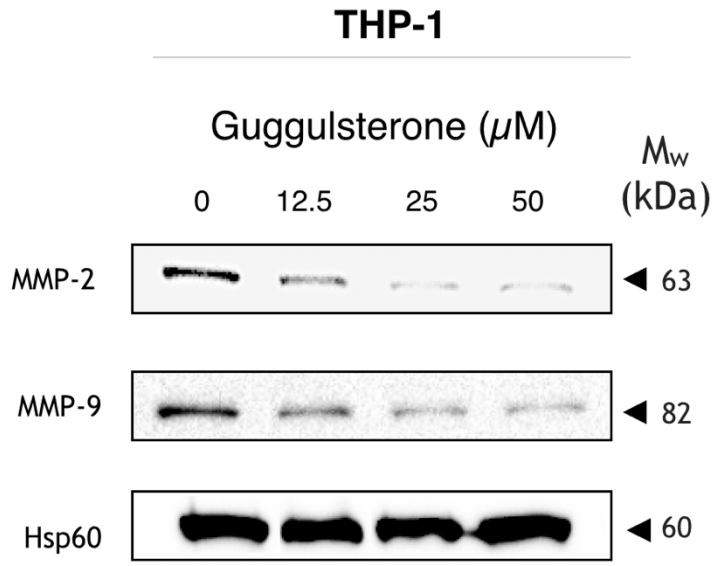

Figure 26. Guggulsterone decreased the expression of MMP-9 in THP1 cell lines. Cells were treated with increasing doses of guggulsterone (12.5, 25, 50 μM) for 48 hrs. Cells were lysed and immunoblotted with antibodies against MMP-2, MMP-9 and HSP-60. Gugglsterone decreased the expression of MMP-2 and MMP-9 in THP1 cells in a dose-dependent manner when treated for 48 hrs. A representative of three independent experiments is depicted in the figure.

5.1.10. Effects of guggulsterone on STAT3 signaling pathway in leukemia cells

STAT3 signaling serves an important role in cell proliferation and survival in many types of cancer including leukemia. Survivin is a STAT3-regulated gene product that is associated with cell proliferation or apoptosis. In order to ascertain whether STAT3 signaling is involved in the anticancer effects of GS on leukemia cells, we used THP1 cells to elaborate the mechanism. The protein expression levels of STAT3, p-STAT3, and survivin were detected by western blotting after treatment of cells with increasing concentrations of GS. The results indicated that GS decreased the phosphorylation of STAT3 but not total STAT3 levels compared with the control group in a dose-dependent manner (Figure 27).

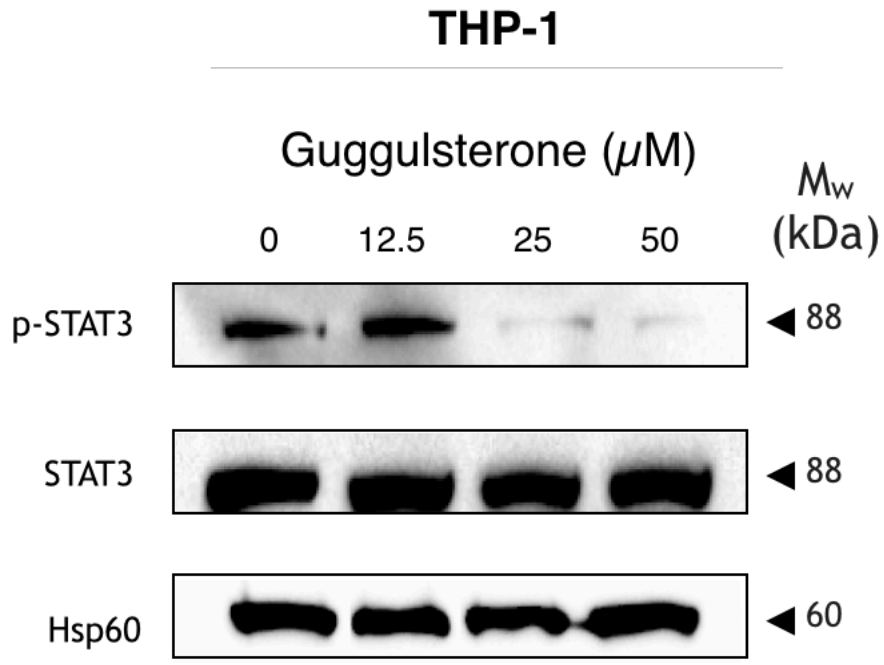

Figure 27. Guggulsterone regulates the expression of STAT3 signaling-related proteins in THP1 cell lines. Cells were treated with increasing doses of guggulsterone (12.5, 25, 50 μM) for 48 hrs. Cells were lysed and immunoblotted with antibodies against p-STAT3, STAT3, and HSP-60. Gugglsterone decreased the expression of p-STAT3 in THP1 cells in a dose-dependent manner when treated for 48 hrs. STAT3 levels did not change with increasing doses of GS. A representative of three independent experiments is shown in the figure.

5.2.Guggulsterone enhances the anti-tumor effects of cisplatin in leukemia cells

In standard treatments today, many chemotherapies are utilized in conjunction with other drugs. To investigate if guggulsterone enhances the anti-cancer activity of other anticancer agents. We chose to evaluate the combinatorial effects with the traditional chemotherapeutic cisplatin.

To properly assess the potential of GS to be used in adjuvant or combination therapies in a novel treatment regimen. Obtained LC50 values were used in designing the plan for combination study. Combination treatment assays were conducted to determine the interactions between GS with cisplatin. Firstly, we tested to effect of combining sub-lethal doses (around the LC50) of Cis and GS on the cellular viability of K562s, THP1, and U937 cells using the CCK-8 viability assay. Results showed that GS significantly fortifies the action of Cis in a synergistic manner in THP1 and U937 cells, but not in K562s (Figures 28-30). Collectively, these results show that GS treatment with Cis leads to strong enhancement of anticancer activity in U937 and THP1 cells. The addition of guggulsterone to cisplatin treatments was able to significantly increase the induction of apoptosis when compared to individual treatments (Table 3). Interestingly, the lowest combination dosage of GS treatment (12.5μM and 25μM) showed comparable inhibition of proliferation to the highest individual treatment dosage of cisplatin (10 μM). This indicates that GS combination treatment was able to show a similar inhibiton of proliferation to individual treatment with a 50-fold decrease in chemotherapeutic cisplatin dose.

5.2.1. Effects of guggulsterone on cell viability in combination with cisplatin in leukemia cells

5.2.1.1. Dose- and time-dependent anti-proliferative effects of guggulsterone on K562s leukemia cells.

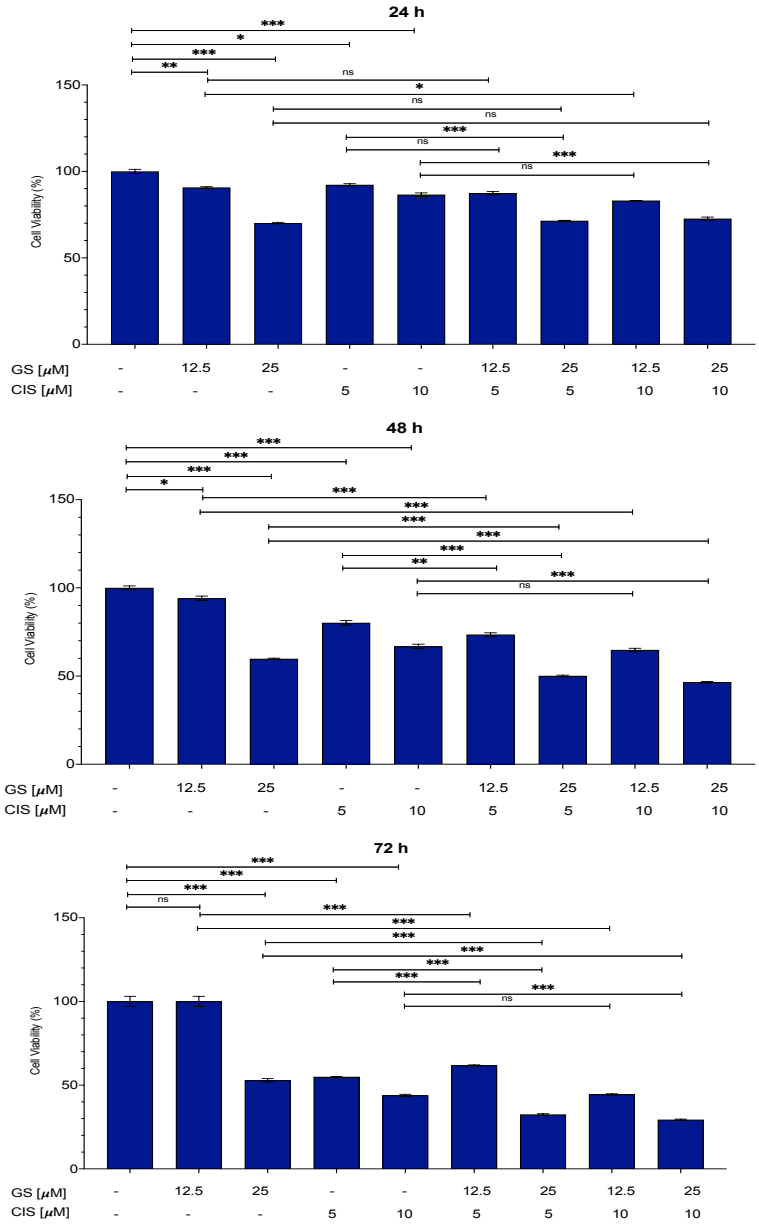

Figure 28. Combination Effect of guggulsterone and cisplatin in K562s cells. K562s cells were treatment with four different combinations of guggulsterone (μM); cisplatin (μM): (12.5; 5), (25; 5), (12.5; 10), (25; 10), for 24 h, 48 h, and 72 hours of incubation as described in the Materials and Methods. Cell viability was measured using CCK-8 assay. Each value is expressed as mean ± SD of three independent experiments, with six technical replicates each. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.2.1.2. Dose- and time-dependent anti-proliferative effects of guggulsterone in U937 leukemia cells.

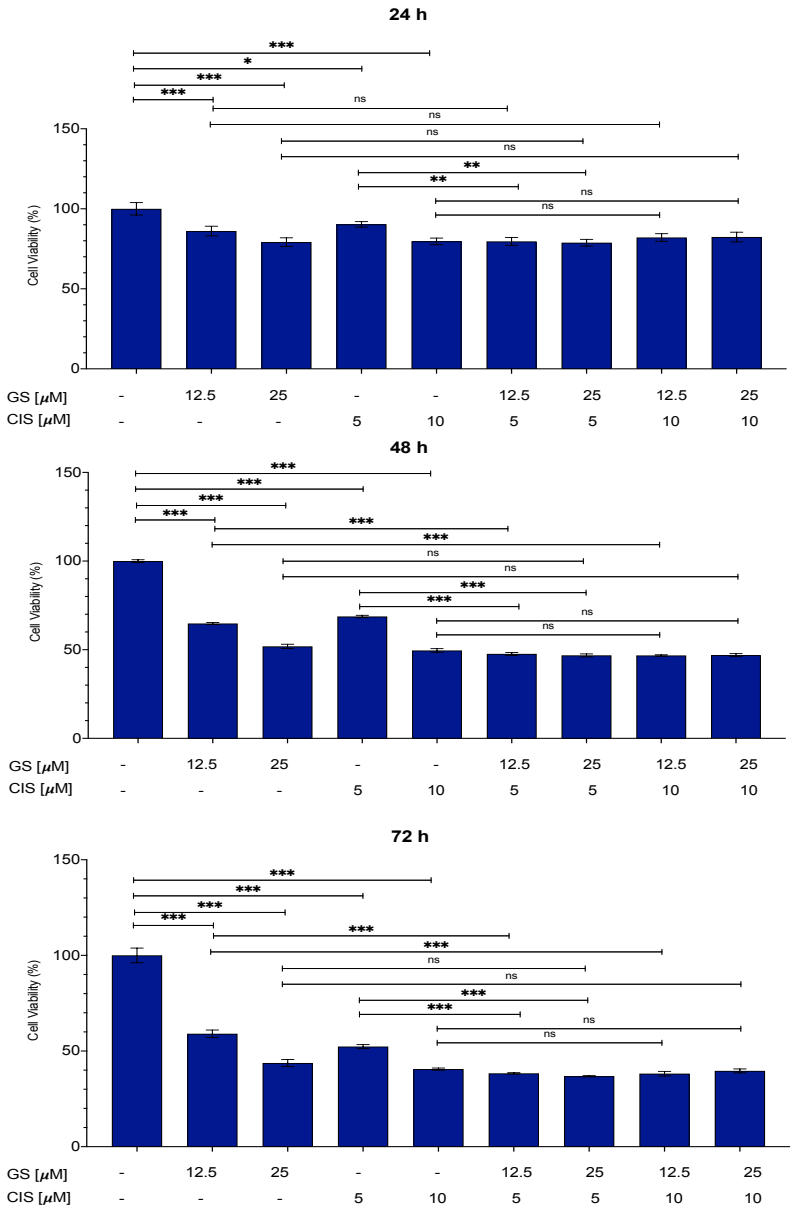

Figure 29. Combination Effect of guggulsterone and cisplatin in U937 cells. U937 cells were treatment with four different combinations of guggulsterone (μM); cisplatin (μM): (12.5; 5), (25; 5), (12.5; 10), (25; 10), for 24 h, 48 h, and 72 hours of incubation as described in the Materials and Methods. Cell viability was measured using CCK-8 assay. Each value is expressed as mean ± SD of three independent experiments, with six technical replicates each. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.2.1.3. Dose- and time-dependent anti-proliferative effects of guggulsterone in THP1 leukemia cells.

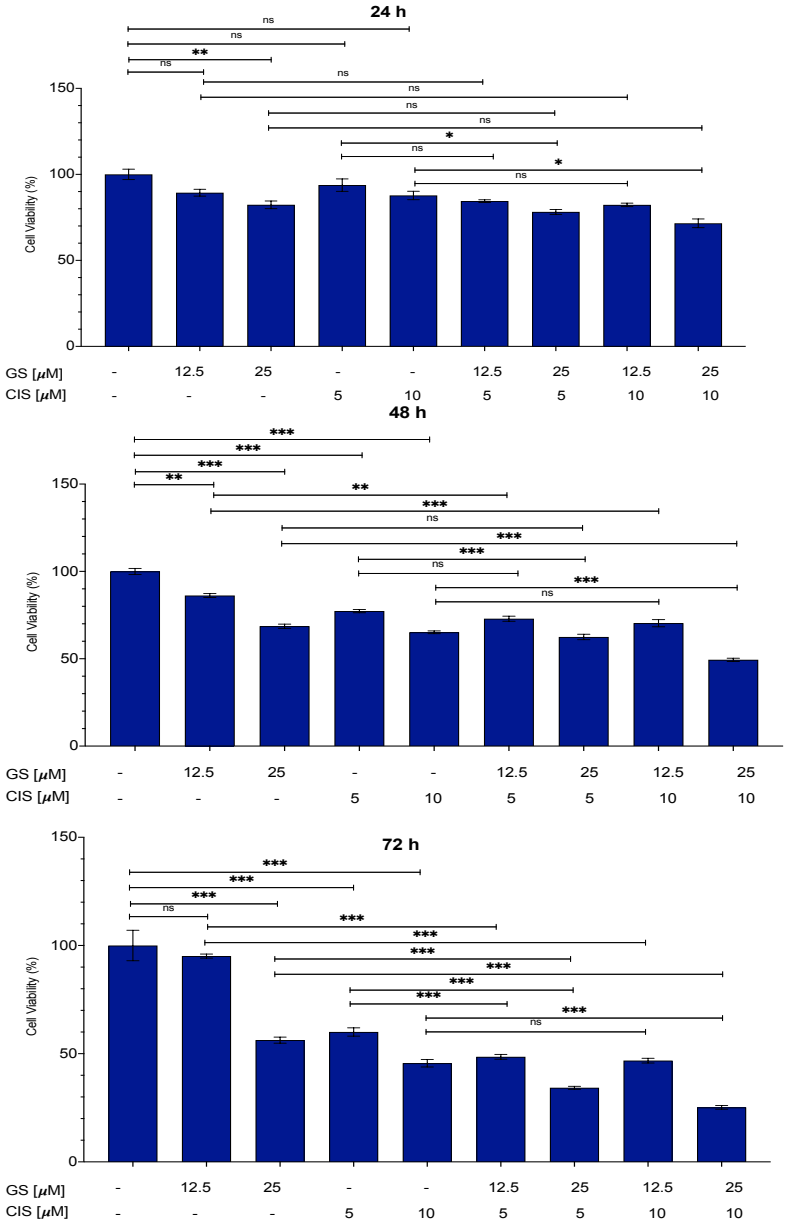

Figure 30. Combination Effect of guggulsterone and cisplatin in THP1 cells. THP1 cells were treatment with four different combinations of guggulsterone (μM); cisplatin (μM): (12.5; 5), (25; 5), (12.5; 10), (25; 10), for 24 h, 48 h, and 72 hours of incubation as described in the Materials and Methods. Cell viability was measured using CCK-8 assay. Each value is expressed as mean ± SD of three independent experiments, with six technical replicates each. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.2.2. Guggulsterone synergistically enhanced the anti-proliferative activity of cisplatin in THP1 and U937 acute leukemia cells but not in K562s chronic leukemia cells.

To determine the effect of the combination, cells were treated with two low doses of guggulsterone (12.5 and 25 μM) and cisplatin (5 and 10 μM) in different combinations of GS; Cis [12.5; 5], [25; 5], [12.5; 10], and [25, 10] and incubated for 24, 48, and 72 h. The growth inhibitory effect of GS and CIs on K562s, THP1, and U937 cells was evaluated by CCK-8 assay. To determine the degree of drug interaction, the fractional index (fa), drug reduction index (DRI), and the combination index (CI) values were calculated using Compusyn software. Figures 31-35 represent the data generated by the Compusyn software analysis. The combination index plots are shown in Figure 31, CI<1, =1, and >1 indicates synergism, additive effect and antagonism, respectively. DRI=1, >1, and <1 indicates no dose-reduction, favorable dose-reduction, and not favorable dose-reduction, respectively, for each drug in the combination. As demonstrated, GS in combination with Cis exhibited synergistic effects on THP1 and U937 cells, but not K562s. Table 3 summarized the Compusyn analysis report for all three cell lines after treatment with four different combinations of GS and Cis for 24, 48, and 72 hrs. To determine the combination ratio and schedule of administration that yield the greatest synergistic effect, a heat map was generated, with the red color highlighting the lowest CI values signifying synergism, white highlighting values near 1 signifying additivity, and blue highlighting values greater than 1 signifying antagonism. CI < 0.1, CI 0.1-0.3, CI 0.3-0.7, CI 0.7-0.85, CI 0.85- 0.9 and CI 0.90-1.10 indicate very strong synergism, strong synergism, synergism, moderate synergism, slight synergism, and nearly additive effects [42,43]. As shown in Table 3, the

combination of GS and Cis yielded a synergistic effect in THP1 and U937 cell lines at a broad concentration range from LC50 to LC90. However, no synergistic effect was observed in K562s. Furthermore, The GS/Cis combination in THP1 cells showed synergy at 24, 48, and 72 h, while in U937, synergy was obtained at 48 h and 72 h only (Table 3, Figure 31-35).

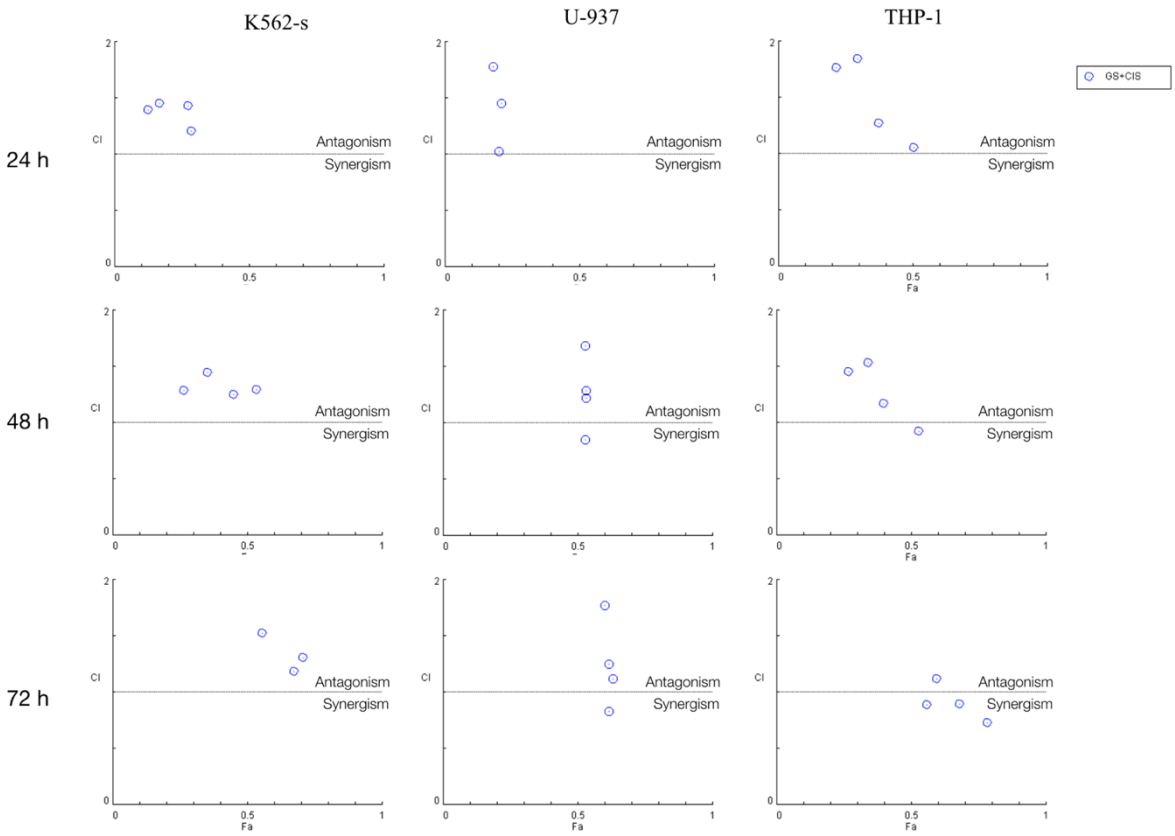

Figure 31. Combination index plots plots for non-constant ratio combinations of guggulsterone and cisplatin after treatment of K562s, U937, and THP1 cells for 24 h, 48 h, and 72 h as described in the Materials and Methods. Cell viability was measured using CCK-8 assay, and CompuSyn software was used to generate the plots. The blue circles represent the CI of GS+Cis combinations as generated by CompuSyn. CI: combination index. CI=1, <1 and >1 indicate additive effect, synergism and antagonism, respectively.

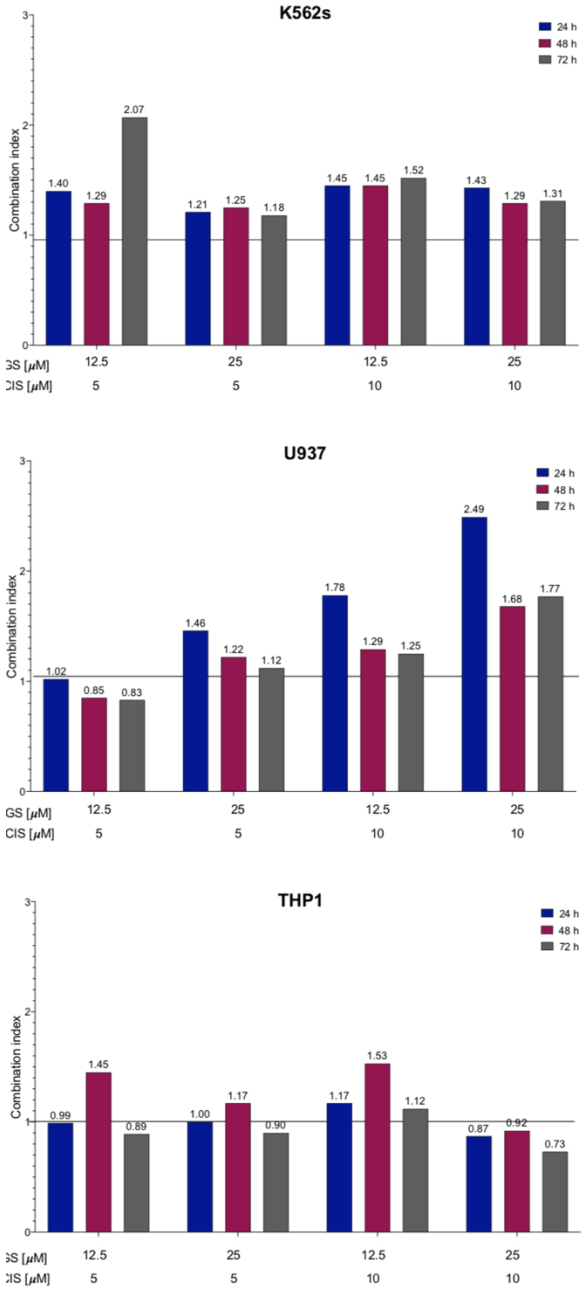

Figure 32. Histogram representation of the combination indices for non-constant ratio combinations of guggulsterone and cisplatin after treatment of K562s, U937, and THP1 cells for 24 h, 48 h, and 72 h as described in the Materials and Methods. Cell viability was measured using CCK-8 assay, and Compusyn software was used to compute the combination indices [CI]. CI=1, <1 and >1 indicate additive effect, synergism and antagonism, respectively.

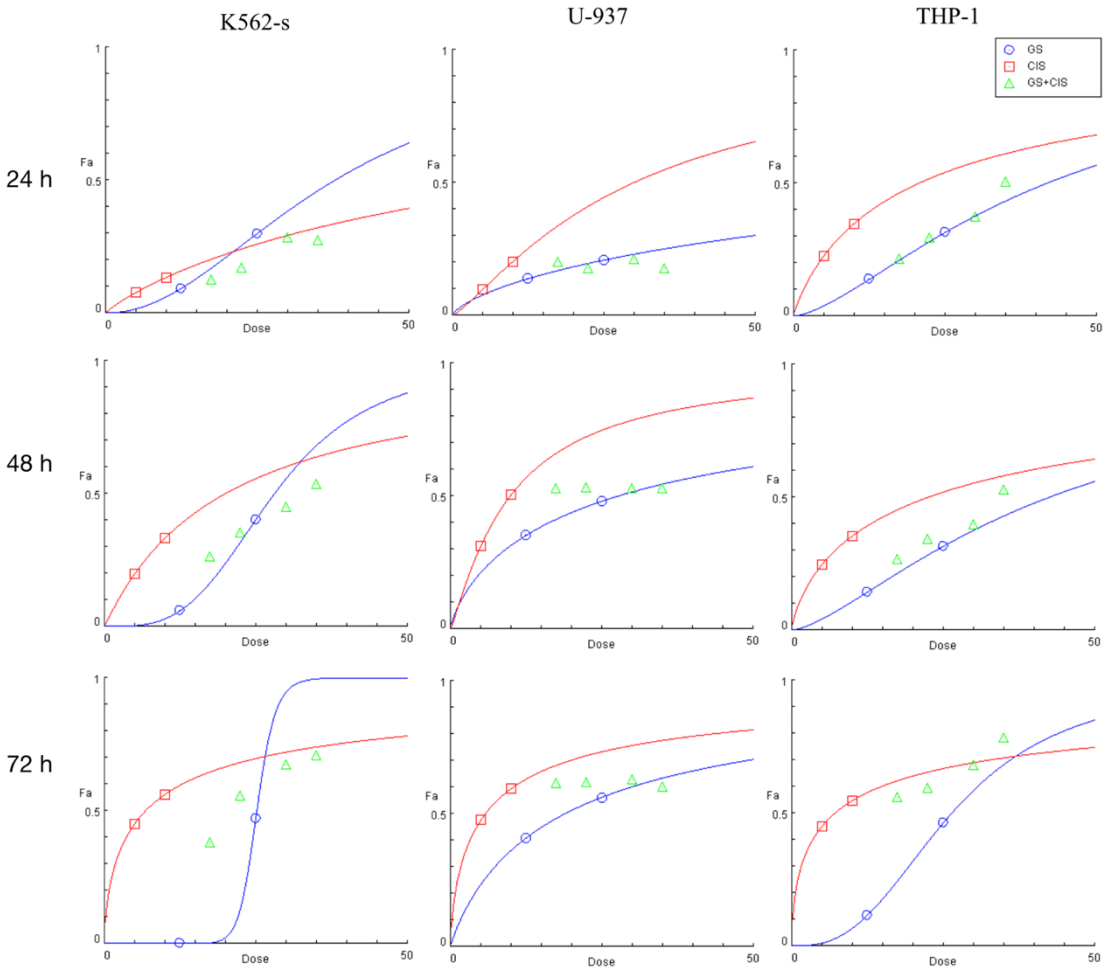

Figure 33. Dose-Effect Curves for non-constant ratio combinations of guggulsterone and cisplatin after treatment of K562s, U937, and THP1 cells for 24 h, 48 h, and 72 h as described in the Materials and Methods. Cell viability was measured using CCK-8 assay, and Compusyn software was used to generate the plots. Fa indicates the fraction of cells affected/killed.

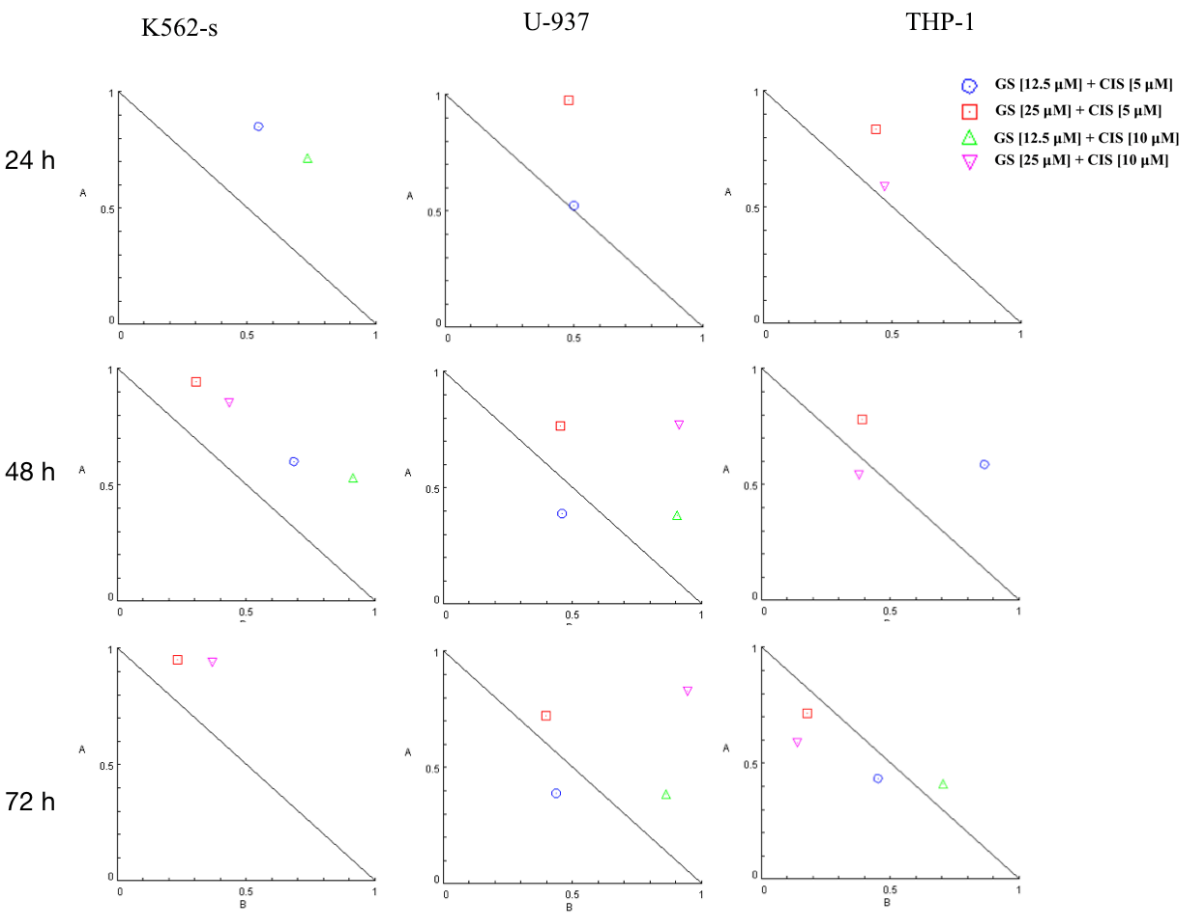

Figure 34. Isobologram plots for non-constant ratio combinations of guggulsterone and cisplatin after treatment of K562s, U937, and THP1 cells for 24 h, 48 h, and 72 h as described in the Materials and Methods. Cell viability was measured using CCK-8 assay, and Compusyn software was used to generate the plots. The combination effects can be summarized as follows: CI<1, dots located lower left; CI=1, dots on the hypotenuse; and CI>1, dots located upper right; these results indicate synergistic, additive, and antagonistic effects, respectively.

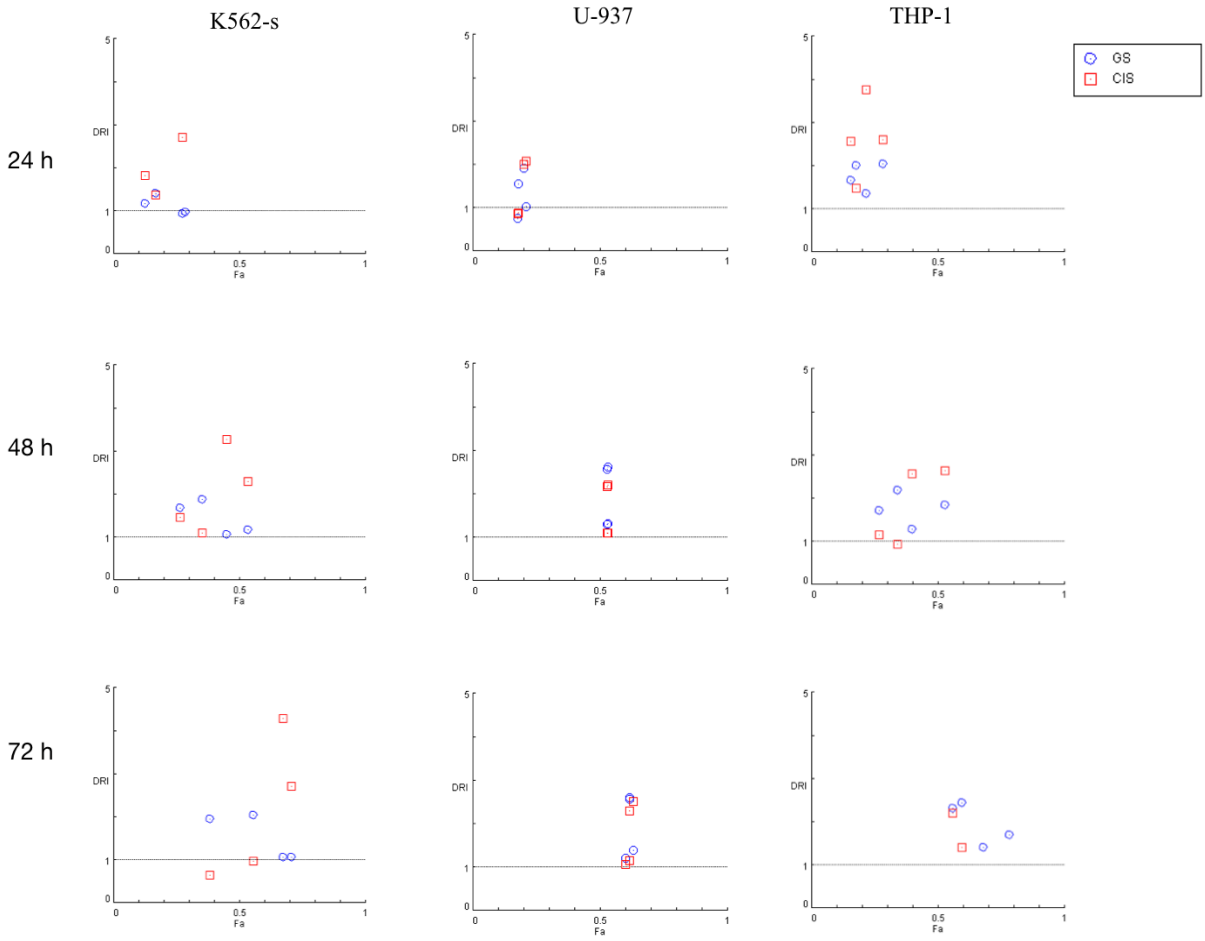

Figure 35. Dose reduction Index plots for non-constant ratio combinations of guggulsterone and cisplatin after treatment of K562s, U937, and THP1 cells for 24 h, 48 h, and 72 h as described in the Materials and Methods. Cell viability was measured using CCK-8 assay, and Compusyn software was used to generate the plots.

Table 4. Combination and dose reduction indices applying combinations of GS and Cis in K562s, U937, and THP1 cell lines.

Time of treatment (hrs)	Cell line	Combination	(Fa)	CI	DRI	
					GS	CIS
24	K562s	GS+CIS[a]	0.13	1.40	1.17	1.83
		GS+CIS[b]	0.29	1.21	0.97	5.78
		GS+CIS[c]	0.17	1.45	1.40	1.36
		GS+CIS[d]	0.27	1.43	0.94	2.70
	U937	GS+CIS[a]	0.20	1.02	1.92	2.00
		GS+CIS[b]	0.21	1.46	1.03	2.08
		GS+CIS[c]	0.18	1.78	1.54	0.88
		GS+CIS[d]	0.18	2.49	0.75	0.87
	THP1	GS+CIS[a]	0.16	0.99	1.66	2.56
		GS+CIS[b]	0.22	1.00	1.35	3.77
		GS+CIS[c]	0.18	1.17	2.00	1.48
		GS+CIS[d]	0.28	0.87	2.04	2.61
48	K562s	GS+CIS[a]	0.27	1.29	1.67	1.46
		GS+CIS[b]	0.45	1.25	1.06	3.26
		GS+CIS[c]	0.35	1.45	1.88	1.09
		GS+CIS[d]	0.54	1.29	1.17	2.30
	U937	GS+CIS[a]	0.53	0.85	2.57	2.17
		GS+CIS[b]	0.53	1.22	1.31	2.19
		GS+CIS[c]	0.53	1.29	2.63	1.10
		GS+CIS[d]	0.53	1.68	1.30	1.09
	THP1	GS+CIS[a]	0.27	1.45	1.71	1.15
		GS+CIS[b]	0.40	1.17	1.28	2.56

Time of treatment (hrs)	Cell line	Combination	(Fa)	CI	DRI	
					GS	CIS
		GS+CIS[c]	0.34	1.53	2.17	0.93
		GS+CIS[d]	0.53	0.92	1.84	2.64
72	K562s	GS+CIS[a]	0.38	2.07	1.96	0.64
		GS+CIS[b]	0.68	1.18	1.05	4.28
		GS+CIS[c]	0.56	1.52	2.04	0.97
		GS+CIS[d]	0.71	1.31	1.06	2.71
	U937	GS+CIS[a]	0.62	0.83	2.57	2.29
		GS+CIS[b]	0.63	1.12	1.38	2.51
		GS+CIS[c]	0.62	1.25	2.59	1.16
		GS+CIS[d]	0.60	1.77	1.21	1.06
	THP1	GS+CIS[a]	0.56	0.89	2.31	2.20
		GS+CIS[b]	0.68	0.90	1.40	5.58
		GS+CIS[c]	0.59	1.12	2.43	1.41
		GS+CIS[d]	0.78	0.73	1.69	7.17

Fa = fractional inhibition; CI = combination index; DRI = drug reduction index. a = GS [12.5 μM] + CIS [5 μM]; b = GS [25 μM] + CIS [5 μM]; c = GS [12.5 μM] + CIS [10 μM]; d = GS [25 μM] + CIS [10 μM].

5.2.3. Effect of guggulsterone/cisplatin combination on apoptosis in leukemia cell lines

Based on the above results, we hypothesized that GS may potentiate the anti-tumor effect of Cis in inducing apoptosis in leukemia cells. To verify whether the decreased cell viability of leukemia cells treated with GS and Cis alone, and in combination was related to apoptosis, flow cytometry utilizing Annexin V-FITC staining and propidium iodide accumulation was used to differentiate early apoptotic cells from living cells as described in the methods section. THP1, and U937 cells were treated with 25uM guggulsterone and/or 10uM cisplatin for a period of 48 hours and incubated with Annexin- V FITC conjugate. Percent positive staining was obtained from a population of 20, 000 cells.

5.2.3.1. Effect of *guggulsterone*/cisplatin combination on apoptosis in U-937 leukemia cell lines

To further confirm whether the synergistic effects of GS and Cis cotreatment on U937 cells were associated with the induction of apoptosis, Annexin V/PI double staining was used to detect apoptosis of U937 cells, which were treated with GS, Cis and their combination. The proportions of early and late apoptotic cells were quantified using flow cytometric analysis, after labeling cells with PI and Annexin V. As shown in Figure 36, there was a marked increase in the number of apoptotic cells when U937 cells were treated with GS or Cis. The results indicated that BR and CDDP, either individually or in combination, were able to generate a significant increase in the apoptotic population of U937 ($P<0.01$). Compared with the GS or Cis groups, a significantly greater apoptotic rate was observed in the GS and Cis cotreatment group ($P<0.01$; Figure 36). Treatment with 25 μM GS resulted in a 15.85% positive Annexin-V staining, which is a significant increase over control cells. Furthermore, treatment with 5 μM cisplatin resulted in a significant 18.55% positive Annexin-V staining in U937 cells and the combined treatment of 25 μM guggulsterone and 5 μM cisplarin resulted in 23% Annexin-V positive, which is significantly more than each of the drugs alone, thus, confirming the hypothesis that GS enhances the anti-tumor effect of Cis when used in combination.

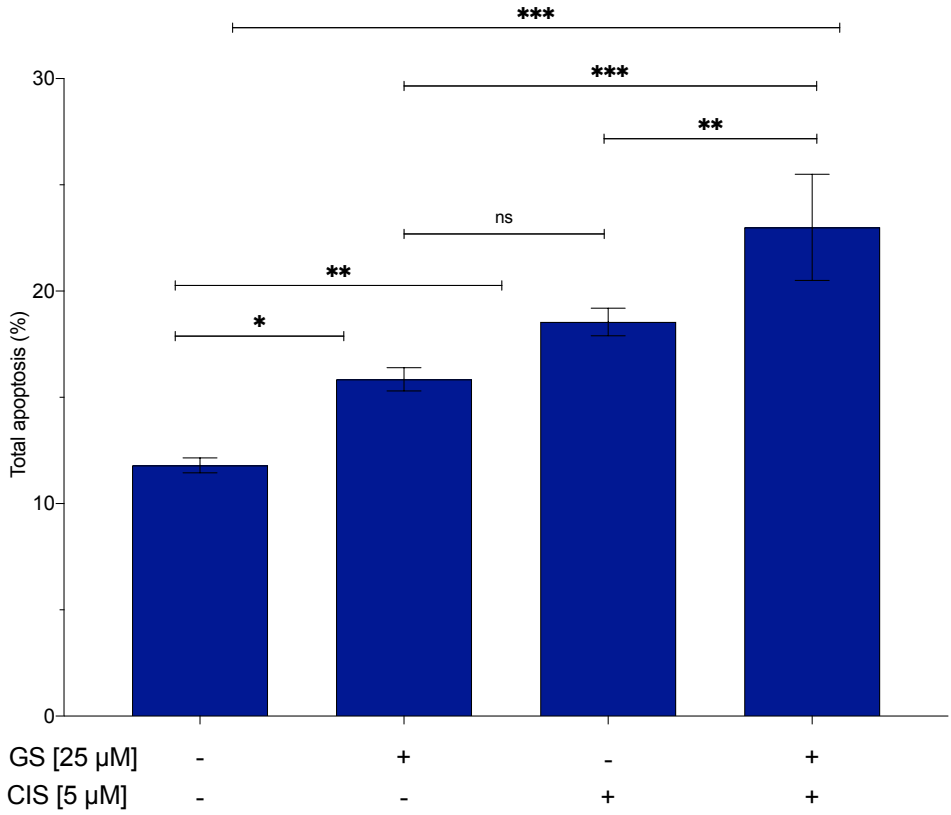

Figure 36. The effects of guggulsterone and cisplatin alone, and in combination on U937 cell growth and apoptosis. Histogram representation of the quantitative percentage of total apoptosis (%) of THP1 cells. The cells were treated with guggulsterone (25 µM) in combination with cisplatin (5 µM) for 48 h, labelled with FITC annexin V and PI, and analyzed by flow cytometry. Each value is expressed as mean ± SD of three measurements. Each value is expressed as mean ± SEM of three independent experiments, with six technical replicates each. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.2.3.2. Effect of *guggulsterone*/cisplatin combination on apoptosis in THP-1 leukemia cell lines

To further investigate whether the synergistic effects of GS and Cis cotreatment on THP1 cells were associated with the induction of apoptosis, Annexin V/PI double staining was used to detect apoptosis of THP1 cells, which were treated with GS, Cis and their combination. The proportions of early and late apoptotic cells were quantified using flow cytometric analysis, after labeling cells with PI and Annexin V. As shown in Figure 37, there was a marked increase in the number of apoptotic cells when THP1 cells were treated with GS or Cis. The results indicated that GS and Cis, either individually or in combination, were able to generate a significant increase in the apoptotic population of THP1 ($P<0.01$). Compared with the GS or Cis groups, a significantly greater apoptotic rate was observed in the GS and Cis cotreatment group ($P<0.01$; Figure 37). Treatment with 25 μM GS resulted in a 13.8% positive Annexin-V staining, which is a significant increase over control cells. Furthermore, treatment with 10 μM cisplatin resulted in a significant 14.5% positive Annexin-V staining in THP1 cells and the combined treatment of 25 μM guggulsterone and 10 μM cisplarin resulted in 22.6% Annexin-V positive, which is significantly more than each of the drugs alone, thus, confirming the hypothesis that GS enhances the anti-tumor effect of Cis when used in combination.

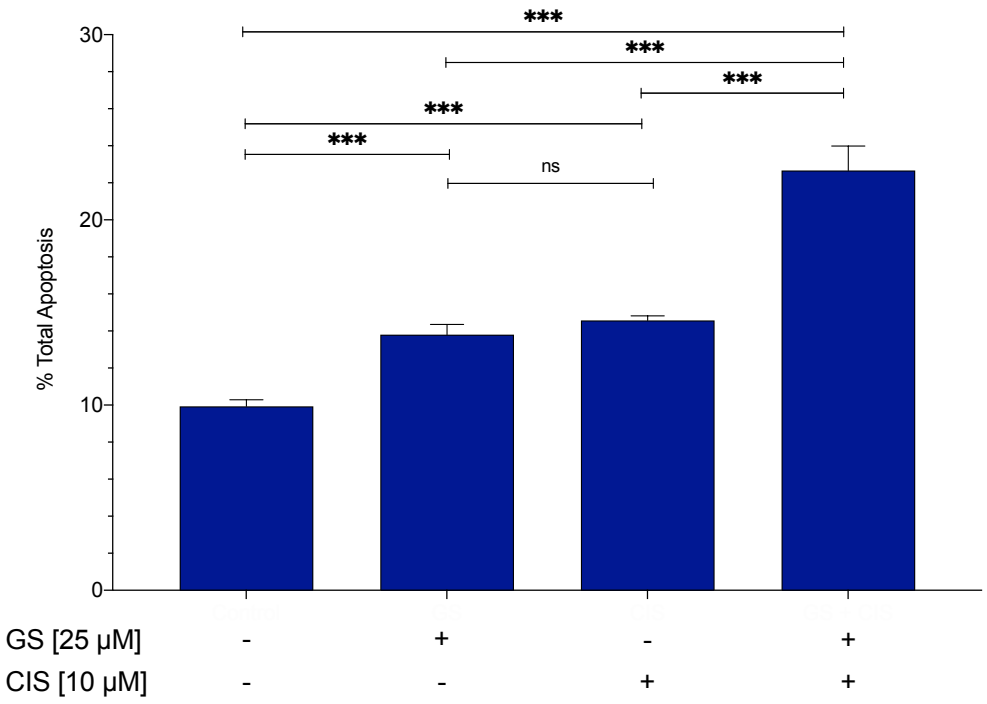

Figure 37. The effects of guggulsterone and cisplatin alone, and in combination on THP1 cell growth and apoptosis. Histogram representation of the quantitative percentage of total apoptosis (%) of THP1 cells. The cells were treated with guggulsterone (25 µM) in combination with cisplatin (10 µM) for 48 h, labelled with FITC annexin V and PI, and analyzed by flow cytometry. Each value is expressed as mean ± SD of three measurements. Each value is expressed as mean ± SEM of three independent experiments, with six technical replicates each. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.2.4. Guggulsterone/cisplatin-induced apoptosis: effect on PARP and caspases in leukemia cells

According to the aforementioned results, the present study aimed to further determine the mechanisms underlying the synergistic antitumor effects of GS and Cis using THP1 cells as a model. Since GS and Cis cotreatment markedly induced apoptosis, the present study focused on the molecular mechanisms underlying apoptosis. In the present study, western blot analysis was used to detect the protein expression levels of caspase-3, caspase-9, and PARP cleavage. As shown in Figure 38, the expression levels of caspase-3 and caspase-9 were markedly decreased following treatment with GS or Cis alone. Compared with in the monotherapy groups, there was a stronger down-regulation of caspase-3 and caspase-9 following GS and Cis cotreatment. Furthermore, GS or Cis alone induced PARP cleavage, and there was a clear enhancement in PARP cleavage following GS and Cis cotreatment compared with in the monotherapy groups.

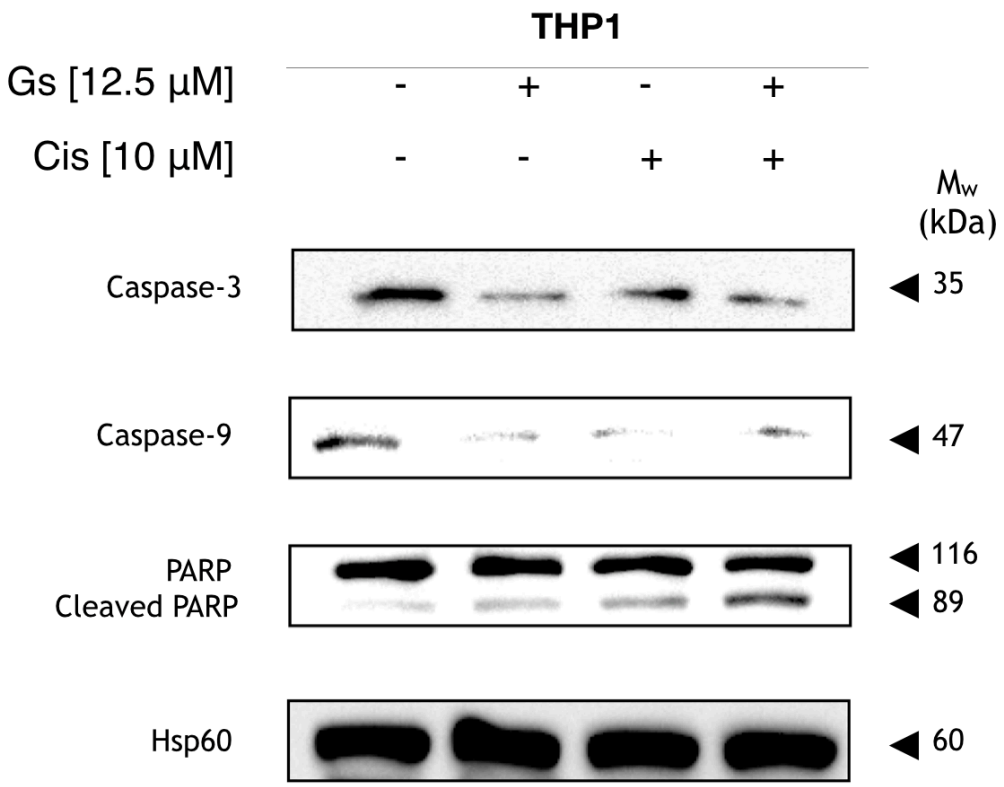

Figure 38. Guggulsterone and cisplatin-induced cleavage of PARP in THP1 cell lines. Cells were treated with guggulsterone (25µM) and cisplatin (10µM) for 48 hrs. Cells were lysed and immunoblotted with antibodies against caspase-3, caspase-9, PARP, and HSP-60. Gugglsterone in combination with cisplatin–induced cleavage of PARP in THP1 leukemia cells when treated for 48 hrs. A representative of three independent experiments is depicted in the figure.

5.2.5. Guggulsterone/cisplatin combination regulate the expression of pro-apoptotic and anti-apoptotic proteins in leukemia cells.

To further explore the synergistic combination effects of guggulsterone in THP1 cells, we studied the expression of apoptosis-regulating proteins of the Bcl-2 family during guggulsterone- and cisplatin-induced apoptosis using western blot analysis on THP1 cell lysates. The expression of pro-apoptotic Bax protein was found to be increased in guggulsterone-induced apoptosis (Figure 39). Treatment with Cis was able to significantly increase the expression levels of Bax ($P<0.01$) and decrease Bcl-2 expression but insignificantly ($P<0.01$). In addition, GS monotherapy significantly increased Bax $P<0.01$), and significantly decreased the expression levels of Bcl-2 ($P<0.01$). There was a significant increase in Bax ($P<0.01$). There was a significant decrease in Bcl-2 after cotreatment with GS/Cis compared to Cis monotherapy, but an insignificant decrease compared to GS monotherapy. Moreover, the Bax/Bcl-2 ratio was also increased significantly following monotherapy or cotreatment, with a more marked increase observed in the cotreatment group compared to monotherapy. Furthermore, bid expression levels were increased. These results indicated that GS and Cis induced cellular apoptosis via a caspase-dependent signaling pathway.

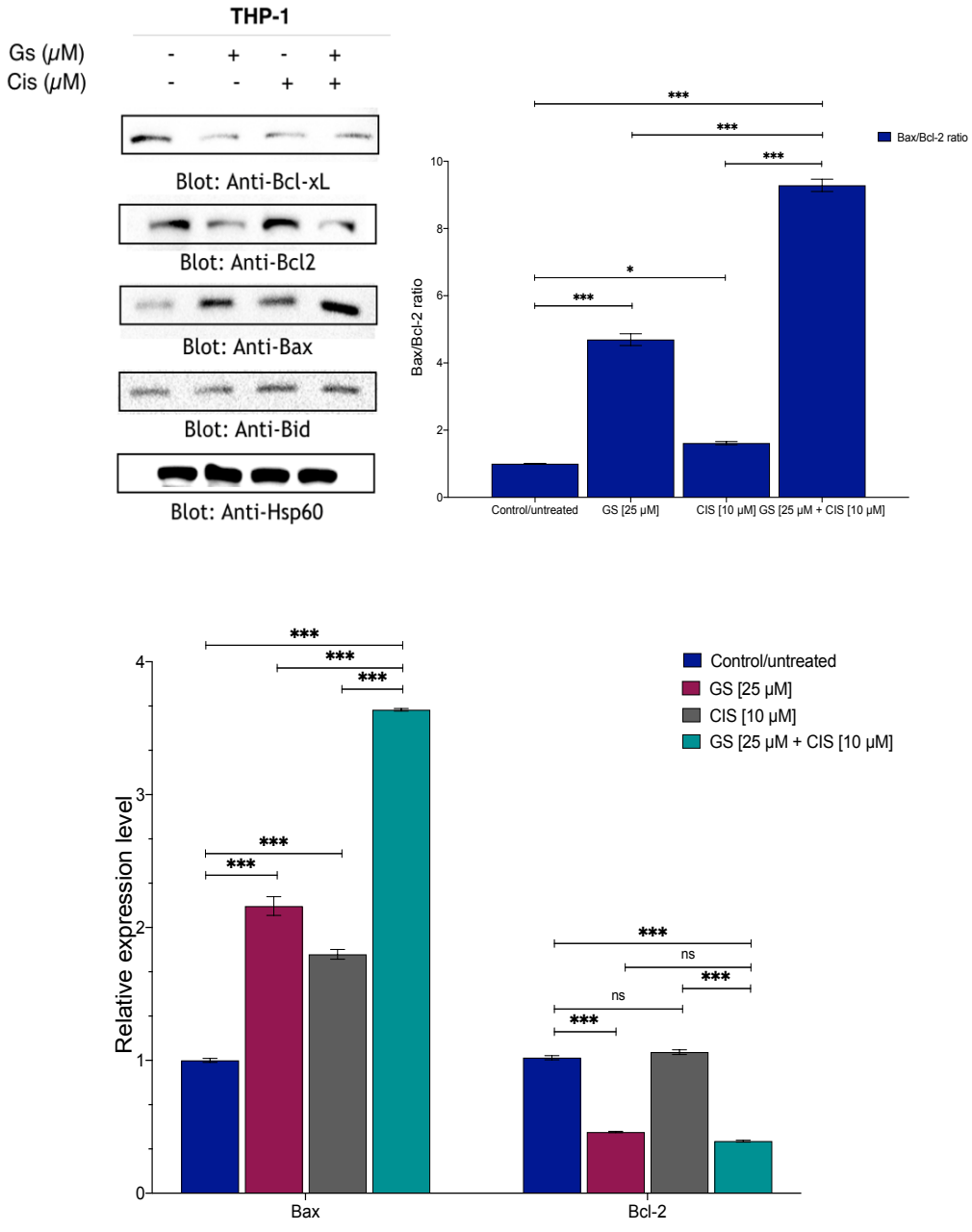

Figure 39. Guggulsterone and cisplatin combination increased the expression of pro-apoptotic proteins and decreased the expression of anti-apoptotic proteins in THP1 cell lines. Cells were treated with guggulsterone (25) and cisplatin (10) for 48 hrs. Cells were lysed and immunoblotted with antibodies against Bax, Bid, Bcl-xl, Bcl-2 and HSP-60. Gugglsterone in combination with cisplatin increased the expression of pro-apoptotic proteins; Bax and Bid, and decreased the expression of anti-apoptotic proteins; Bcl-xl and Bcl-2 in THP1 cell lines in THP1 leukemia cells when treated for 48 hrs. A representative of three independent experiments is shown.

5.2.6. Guggulsterone/cisplatin combination modulate the expression of inhibitor of apoptosis (IAP) proteins in leukemia cell lines.

To further confirm whether the synergistic effects of GS and Cis cotreatment on THP1 cells were associated with the induction of apoptosis, Western blot analysis was used to detect the protein expression levels of xIAP, cIAP-1, and survivin proteins in THP1 cells after treatment with GS, Cis and their combination. As shown in Figure 41, the expression levels of xIAP, cIAP-1, and survivin were markedly decreased following treatment with GS or Cis alone. Although there was a marked reduction in the expression of xIAP, cIAP-1, and survivin compared to control, there was a weaker down-regulation of the proteins compared with in the monotherapy groups, indicating no prominent involvement of xIAP, cIAP-1, or survivin in the synergistic effect of the GS/Cis combination.

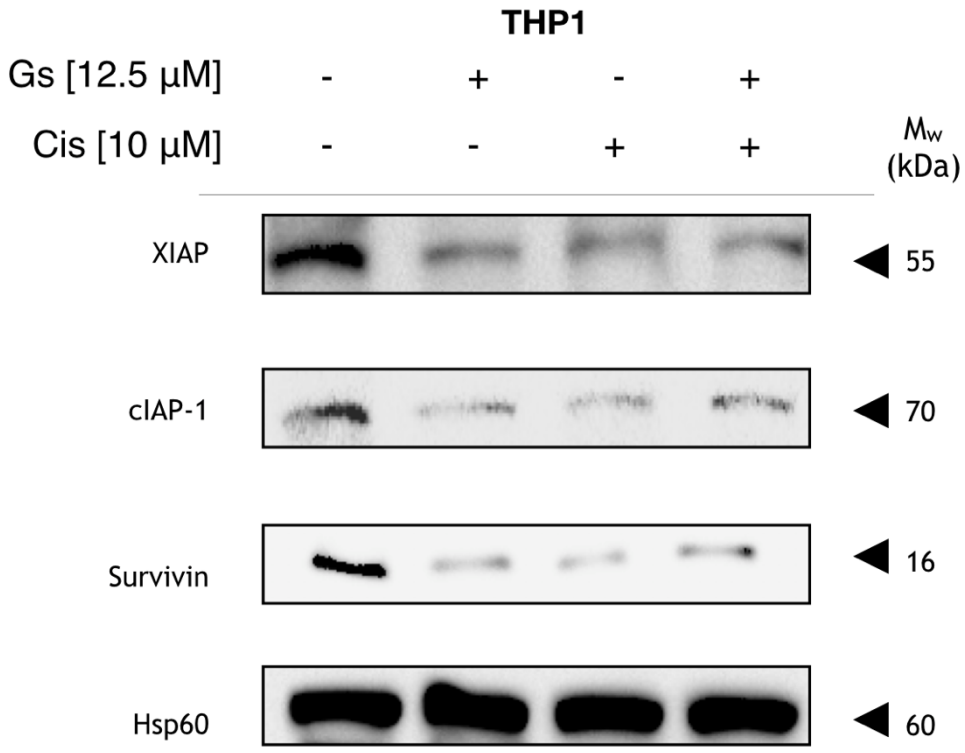

Figure 41. Effect of guggulsterone and cisplatin combination on the expression of the intracellular apoptosis inhibitor proteins xIAP and cIAP-1 in THP1 cell lines. Cells were treated with guggulsterone (25 µM) and cisplatin (10 µM) for 48 hrs. Cells were lysed and immunoblotted with antibodies against XIAP, cIAP-1, survivin and HSP-60. A representative of three independent experiments is depicted in the figure.

5.2.7. Effect of guggulsterone/cisplatin combination on cell cycle distribution in leukemia cells

5.2.7.1. Effect of guggulsterone/cisplatin combination on cell cycle distribution in U937 leukemia cells

The cell cycle phase distribution of U937 cells treated with GS at 48 hours is depicted in Figure 42. As shown in the figure, the pattern of distribution at different phases for U937 was significantly altered in a dose-dependent manner after incubation with increasing doses of GS for 48 h. Following GS treatment, s significant reduction of U937 cells at G0/G1 was found following monotherapy, however, although following GS/Cis co-treatment there was a noticeable reduction in the % of cells in G0/G1 compared, but there was no reduction compared to monotherapy. In addition, there was a concomitant significant increase in sub G0/G1 phase after monotherapy, with Cis causing a more significant increase in the subG0/G1 population compared to GS ($P<0.01$). There was, no apparent signs of cell cycle arrest and only small but statistically insignificant variation were observed in all other phases of the cell cycles, suggesting cell death mechanisms were involved in the anti-tumorigenic action of GS/Cis in combination.

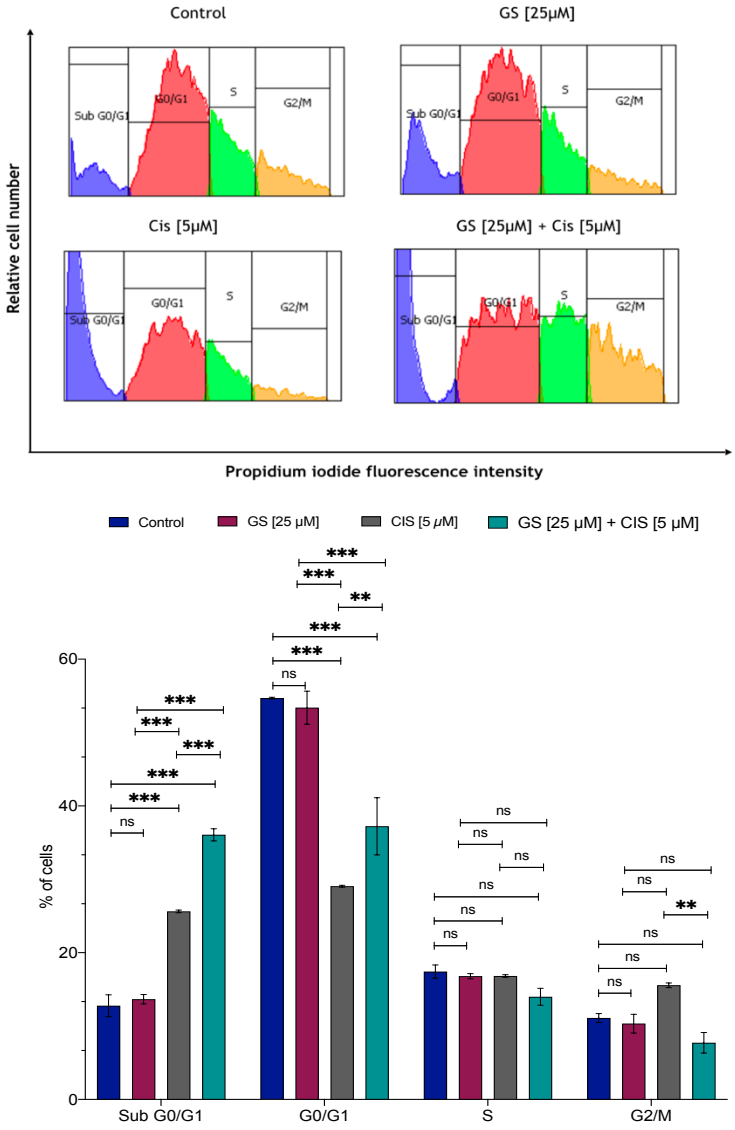

Figure 42. Effects of guggulsterone/cisplatin combination on cell cycle distribution of U937 cells. (A) Representative graphs obtained by flow cytometric analysis representing relative cell number at the different phases following treatment with GS and Cis alone/and in combination. (B) Representative histograph of the dose-dependent effect of GS on the cell cycle distribution of U937 cells. Cell population percentages of sub-G0/G1, G0/G1, S and G2/M phases are indicated in the figure. Statistical analyses are shown as averages with indicated standard errors (n = 3). The values represent the mean ± SEM from three independent samples. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.2.7.2.Effect of guggulsterone/cisplatin combination on cell cycle distribution in THP1 leukemia cells

The cell cycle phase distribution of THP1 cells treated with GS at 48 hours is depicted in Figure 43. As shown in the figure, the pattern of distribution at different phases for THP1 was significantly altered in a dose-dependent manner after incubation with increasing doses of GS for 48 h. Following GS treatment, s significant reduction of THP1 cells at G0/G1 was found following monotherapy, however, although following GS/Cis co-treatment there was a noticeable reduction in the % of cells in G0/G1 compared, but there was no reduction compared to monotherapy. In addition, there was a concomitant significant increase in sub G0/G1 phase after monotherapy, with Cis causing a more significant increase in the subG0/G1 population compared to GS ($P<0.01$). There was, no apparent signs of cell cycle arrest and only small but statistically insignificant variation were observed in all other phases of the cell cycles, suggesting cell death mechanisms were involved in the anti-tumorigenic action of GS/Cis in combination.

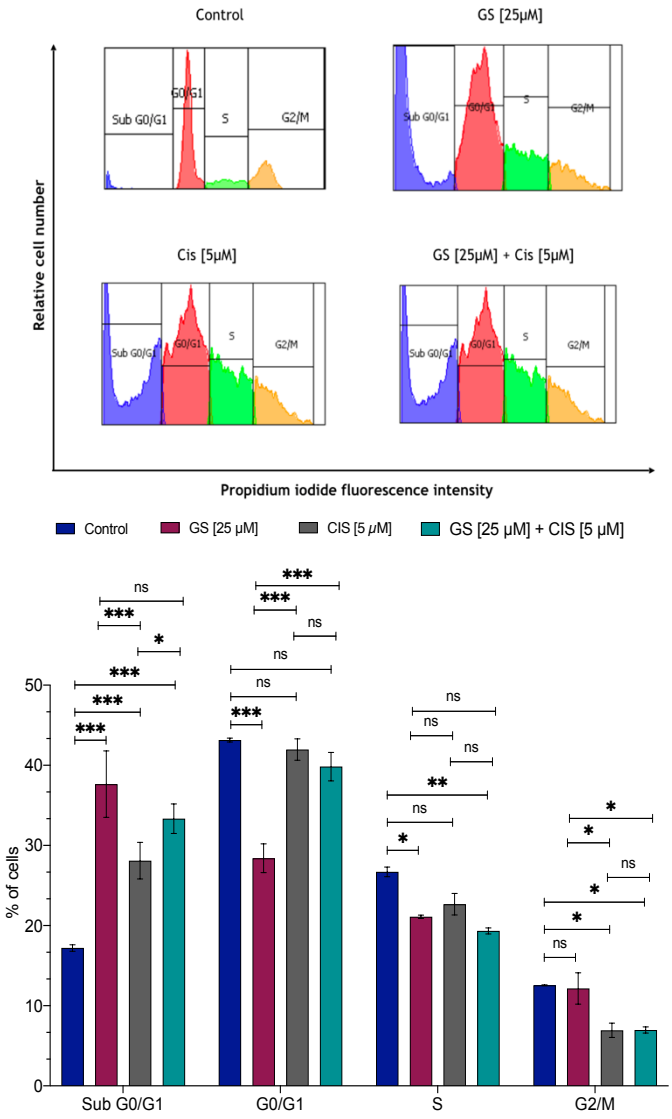

Figure 43. Effects of guggulsterone/cisplatin combination on cell cycle distribution of THP1 cells. (A) Representative graphs obtained by flow cytometric analysis representing relative cell number at the different phases following treatment with GS and Cis alone/and in combination. (B) Representative histograph of the dose-dependent effect of GS on the cell cycle distribution of THP1 cells. Cell population percentages of sub-G0/G1, G0/G1, S and G2/M phases are indicated in the figure. Statistical analyses are shown as averages with indicated standard errors (n = 3). The values represent the mean ± SEM from three independent samples. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant.

5.2.8. *Effect of guggulsterone/cisplatin combination on the expression of mitochondrial integrity proteins in leukemia cells*

THP1 cell lines were treated with increasing doses of GS and Cis alone/and in combination with the indicated concentrations for 48 h, lysed and immunoblotted with antibodies against MMP-2, MMP-9, and HSP-60. As depicted in Figure 26, treatment with GS for 48 h suppressed the expression of MMP-2 and MMP-9 in THP1 more than each drug alone (Figure 44).

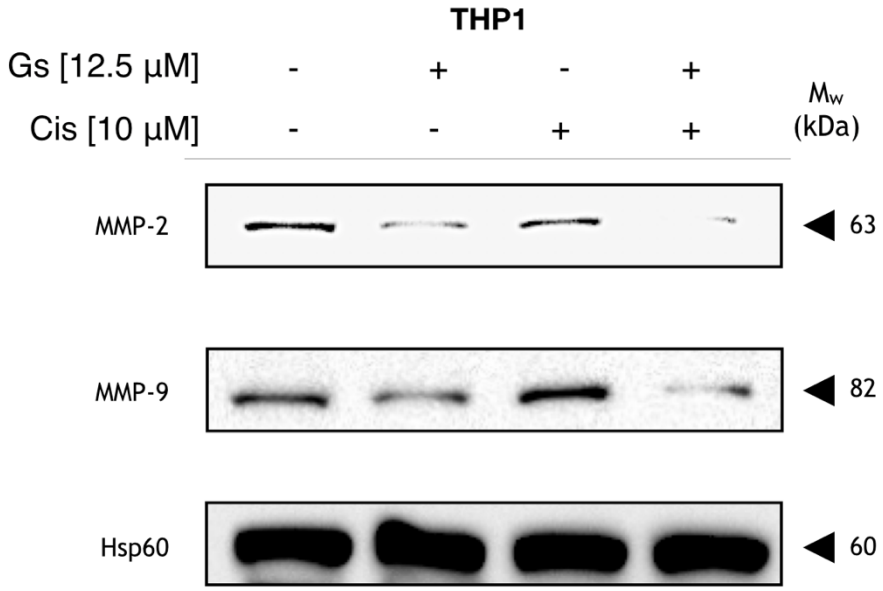

Figure 44. Guggulsterone decreased the expression of MMP-9 and MMP-2 in THP1 cell lines. Cells were treated with increasing doses of guggulsterone and cisplatin alone/and in combination for 48 hrs. Cells were lysed and immunoblotted with antibodies against MMP-2, MMP-9 and HSP-60. Gugglsterone/cisplatin combination decreased the expression of MMP-2 and MMP-9 in THP1 cells when treated for 48 hrs. A representative of three independent experiments is depicted in the figure.

5.2.9. Effect of guggulsterone/cisplatin combination on STAT3 signaling pathway in leukemia cells

STAT3 signaling serves an important role in cell proliferation and survival in many types of cancer including leukemia. In order to ascertain whether STAT3 signaling is involved in the anticancer effects of GS on leukemia cells, for cell culture convenience, we used THP1 cells to elaborate the mechanism. The protein expression levels of STAT3 and p-STAT3 were detected by western blotting after treatment of cells with increasing concentrations of GS. The results indicated that GS greatly decreased the phosphorylation of STAT3 and total STAT3 levels in the GS/Cis combination group compared to monotherapy (Figure 45).

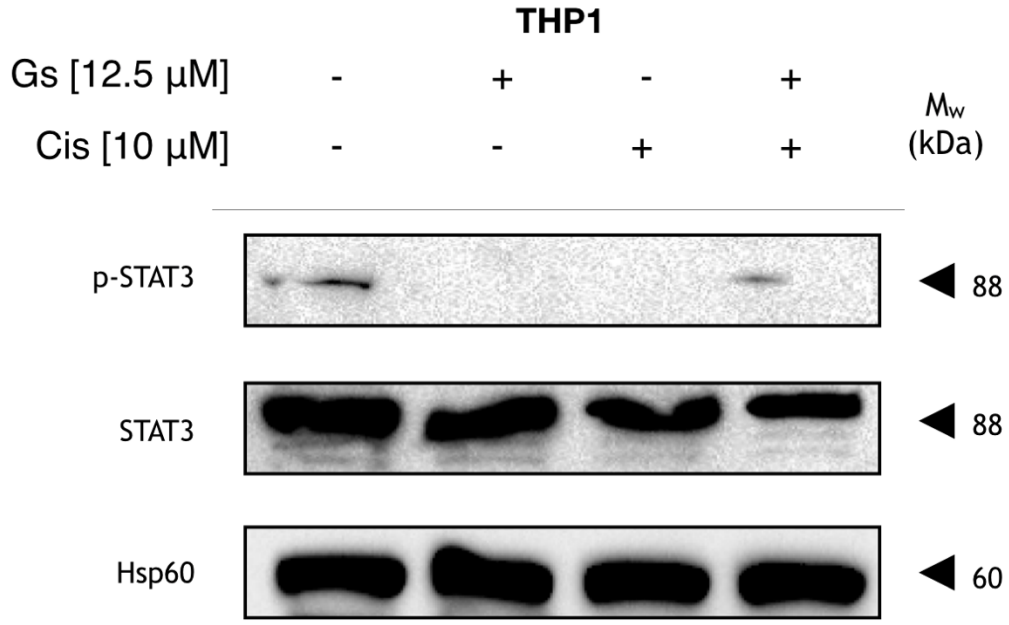

Figure 45. Guggulsterone regulates the expression of STAT3 signaling-related proteins in THP1 cell lines. Cells were treated with guggulsterone and Cis alone/and in combination for 48 hrs. Cells were lysed and immunoblotted with antibodies against p-STAT3, STAT3, and HSP-60. Gugglsterone/cisplatin combination decreased the expression of p-STAT3 and STAT3 levels in THP1 cells when treated for 48 hrs. A representative of three independent experiments is shown in the figure.

6. DISCUSSION

Leukemia is considered the most common hematopoietic malignancy. It is one of the most common malignant tumors, particularly in children. It is known to develop and progress in a multifactorial and multi-step process that involves genetic and epigenetic changes, making it one of the most untreatable forms of malignancy. The survival rates for leukemia diagnosis are abysmal, leaving patients hopeless.

The success of current therapies has been extremely limited, and in many cases, completely ineffective. In addition, current therapies often rely on invasive procedures and high doses of one or more chemotherapeutic agents that can lead to major toxicities and collateral side effects. The need for innovative solutions to these problems has never been higher and the search for more effective therapies has illuminated new possibilities and continues to explore beyond the known pathology and treatments. One such promising avenue for research is uncovering the potential for combined and synergistic enhancement of known therapies [90, 91] through the use of natural compounds. A combination therapy that joins traditional chemotherapy with natural compounds, is now considered a new innovative approach for overcoming multidrug resistance and cell toxicity. The main aims are to achieve synergistic therapeutic effect, dose and toxicity reduction, and to minimize or delay the induction of drug resistance. [19].

Phytochemicals have been extensively studied for their cancer therapeutic properties (Øverby, Zhao, & Chen, 2014). Guggulsterone is a one such natural compound studied for its anticancerous and antiproliferative properties as a single agent or in combination (K. S. Ahn, Sethi et al., 2008; An, Cheon et al., 2009; De Gottardi,

Dumonceau et al., 2006; Dixit, Ghildiyal et al., 2013; Guan, Hoque et al., 2014; Guan, Li et al., 2013; Jiang, Xiao et al., 2013; B. H. Kim, Yoon et al., 2013; E. S. Kim, Hong et al., 2008; Kong, He et al., 2015; Krishnamurthy, Wang, Rokhfeld, & Bieberich, 2008; Leo, Therachiyil et al., 2019; C. Li, Zang et al., 2009; Macha, Matta, Chauhan, Siu, & Ralhan, 2010; Macha, Matta, Chauhan, Siu, & Ralhan, 2011b; Macha, Rachagani et al., 2013; Moon, Park, Choi, Ahn, & Kim, 2011; Peng, Raufman et al., 2012; Samudio, Konopleva et al., 2005; J.-J. Shi, X.-L. Jia et al., 2015; Shishodia & Aggarwal, 2004; Shishodia, Sethi et al., 2007; S. V. Singh, S. Choi, Y. Zeng, E.-R. Hahm, & D. Xiao, 2007; S. V. Singh, Zeng et al., 2005; W. C. Wang, Uen et al., 2012; Xiao & Singh, 2008; Xiao, Zeng et al., 2011; H.-B. Xu, Z.-L. Shen, J. Fu, & L.-Z. Xu, 2014; Xu, Li, & Liu, 2011; Yamada, Osawa et al., 2010; Zhong, Yang et al., 2015). Guggulsterone has been previously shown to exhibit selective cytotoxic effects in several types of tumors, mainly solid tumors including pancreatic cancer (D. W. Ahn, Seo et al., 2012; Lv, Song et al., 2008; Macha, Rachagani et al., 2013), esophageal (De Gottardi, Dumonceau et al., 2006; Guan, Hoque et al., 2014; Guan, Li et al., 2013; Yamada, Osawa et al., 2014), colon (An, Cheon et al., 2009; Cheon, Kim et al., 2006; J. M. Kim, Kang et al., 2010; Martinez-Becerra, Monte et al., 2012), breast (Jiang, Xiao et al., 2013; Noh, Chung et al., 2013), prostate (S. V. Singh, Zeng et al., 2005), hepatocellular (Moon, Park et al., 2011; J.-J. Shi, X.-L. Jia et al., 2015), as well as head and neck cancers (Macha, Matta et al., 2011b). Most importantly the safety profile of this drug (Agarwal, Singh et al., 1986; Urizar & Moore, 2003) makes it a good therapeutic agent for complementary and preventive therapy. It has been previously shown that GS exerts no cytotoxic effects in normal PBMCs (Samudio, Konopleva et al., 2005; J. J. Shi, X. L. Jia et al., 2015; S. V. Singh, S. Choi et al., 2007). Although

the effect of guggulsterone has been investigated previously using cell lines derived from solid tumors, the effect in leukemia cells has not been well investigated. In addition, the conclusive mechanisms responsible for its anticancer effects are still not fully elucidated (Bhat, Prabhu et al., 2017).

In the present work, we aimed to determine the efficacy of GS alone, and in combination with the chemotherapeutic agent cisplatin, and to elucidate its mechanism of action using human leukemia cell lines. We investigated the anti-cancer effects of GS through which it could prove beneficial both alone and as an adjunct therapy in targeting leukemia using three different leukemia cell lines; K562s (chronic myelogenous leukemia), U937 (acute myelomonoblastic leukemia), and THP1 (acute monocytic leukemia).

We first conducted a cytotoxicity screening by testing the sensitivity of the three leukemia cell lines to GS alone using CCK-8 viability assay to investigate the effects of both GS on the long-term proliferation in culture. Our results showed that GS is efficient in inhibiting cell proliferation in all three cell leukemia lines in a time- and dose- dependent manner, but different sensitivity patterns were observed. Among the three tested cell lines, U937 cell line was the most sensitive. The differential sensitivity patterns seen within acute (U937 and THP1) and chronic (K562s) cell lines could be due to different cellular origin, genetic abnormalities and or expression of differential proteins by different types of cells. From this data, we were able to determine the LC50 values for guggulsterone in each cell line at each time point (Table 3). A comparison of this data shows that there is a considerable variation between the time points. With this in mind, further assessment including, cell cycle, apoptosis, and protein expression, was completed at 48 h in the three cell lines. GS has been previously shown to strongly

inhibit proliferation in a time- and dose- dependent manner in other cell lines (D. W. Ahn, Seo et al., 2012; K. S. Ahn, Sethi et al., 2008; An, Cheon et al., 2009; Attia, Tawfiq, Ali, & Elmazar, 2017; Guan, Li et al., 2013; Leo, Therachiyil et al., 2019; C. Li, Zang et al., 2009; Macha, Matta et al., 2010; Macha, Matta et al., 2011b; Macha, Rachagani et al., 2013; Peng, Raufman et al., 2012; Samudio, Konopleva et al., 2005; J.-J. Shi, X.-L. Jia et al., 2015; Shishodia & Aggarwal, 2004; Shishodia, Sethi et al., 2007; S. V. Singh, Zeng et al., 2005; Yang, Lee et al., 2012; Zhong, Yang et al., 2015).

GS has been shown to induce apoptosis in a wide range of cancer cells (Jiang, Xiao et al., 2013; R. J. Leeman-Neill, S. E. Wheeler et al., 2009; Macha, Matta, Chauhan, Siu, & Ralhan, 2011a; Macha, Rachagani et al., 2013; Shishodia & Aggarwal, 2004; Shishodia, Sethi et al., 2007; Shivendra V. Singh, Sunga Choi et al., 2007; S. V. Singh, Zeng et al., 2005). Given that it is important for an effective chemotherapeutic agent to cause irreversible death in cancer cells, i.e., apoptosis. We first examined the mechanism by which GS inhibited cell proliferation by examining its effects in modulating the apoptotic pathway in leukemia cells. Our findings indicate that apoptosis contributes, at least in part, to the antiproliferative effects of guggulsterone. The evidence for proapoptotic activity of guggusterone was revealed by increased annexin-V/PI positive cells (Figure 12-14). Interestingly, among all three cell lines, U937 and THP1 acute leukemia cells were more sensitive compared to the chronic leukemia cell line K562s which showed significant but weaker increase in apoptosis 48 h post-treatment (Figure 8-10). This can be explained by the gene expression signature of K562 human chronic myelogenous leukemia cells, which, unlike THP1 and U937 human acute leukemia cells, are relatively resistant to drug-induced apoptosis because, like the majority of human CML, they express the genotypic abnormality involving

dysregulation of the p210 tyrosine kinase activity of the bcr-abl fusion oncoprotein ($p210^{bcr\text{-}abl}$) (Calabretta, 1991). The expression of $p210^{bcr\text{-}abl}$ in K562 cells is known to be responsible for their resistance to differentiation and drug-induced apoptosis (Bedi, Barber et al., 1995; Calabretta, 1991). Thus, it is not surprising to see differential sensitivity patterns between cell lines. Future investigation is needed to understand the molecular mechanisms of GS on leukemia cell lines to elucidate further the molecular targets of GS and hence identify reasons for the selective targeting toward acute leukaemia cell lines.

To further explain the mechanism of guggulsterone and to confirm apoptosis, we investigated the effect of guggulsterone on cell cycle distribution. Interestingly, in addition to induction of apoptosis, GS treatments increased the % of cells in sub G0/G1 population and induced changes in the different phases which was dependent on the type of cells investigated (acute/chronic) and treatment dose (Figure 20-22), suggesting that cell death mechanisms were involved in the anti-tumorigenic action of GS, especially around the dose of LC50 (about 50 μM). Thus, cell cycle arrest can be a useful target for leukemia therapies. These differential sensitivity patterns seen within acute and chronic cell lines could be due to different cellular origin, genetic abnormalities and or expression of differential proteins by different types of cells. GS was previously showed to elicit minute level of cytotoxicity against normal cells, such as prostate epithelial cell lines, normal human fibroblast cell and normal peripheral blood mononuclear cells (Samudio, Konopleva et al., 2005; J. J. Shi, X. L. Jia et al., 2015; S. V. Singh, S. Choi et al., 2007). In addition, it was previously shown that GS treatment does not induce cell cycle arrest and apoptosis in normal cell lines, which may explain the reason why a cell line is more resistant to the growth inhibition by GS.

To test whether guggulsterone was able to physiologically inhibit its intended target, western blot analysis was performed on whole-cell lysates from the three leukemia cells treated for 48 h day with and without GS. This treatment was done to exclude the physiological effect of pathway inhibition (cell death, altered growth profile, etc) from the direct effect of signaling block, to confirm apoptosis, and to further elucidate the mechanism of action of guggulsterone in inducing apoptosis in leukemia. It is well known that cancer invasion and metastasis is a complicated multistep process involving numerous effector molecules. Thus, we tested several cancer biomarker proteins by western blot analysis in all three leukemia cell lines.

Cellular demolition in apoptosis is carried out by caspases, re well known to play a role as key mediators of apoptosis that lead to DNA fragmentation and subsequently cell death, via the cleavage of specific cellular substrates, including PARP, an endogenous substrate of caspase-3, finally causing apoptosis (Lazebnik, Kaufmann, Desnoyers, Poirier, & Earnshaw, 1994; Thornberry, 1998). Our data clearly showed that treatment with GS induced the activation of both caspases-9 and -3 at 48 h in a dose-dependent manner and increases levels of cleaved caspase-3 (Figure 16-18) in all three leukemia cell lines. Furthermore, a progressive proteolytic cleavage of PARP was observed in all three leukemia cell lines, thus clearly indicating that the activation of caspases and PARP cleavage are involved in the GS-induced apoptosis. These data indicate that caspases are the key molecules mediating GS-induced apoptosis, and GS-induced apoptosis in K562s, THP1, and U937 cells may be mediated through a caspase-dependent pathway. This is consistent with Shishodia *et al.* (2007) who showed that GS-induced apoptosis in five human leukemia cell lines involved the activation of caspases and PARP cleavage (Shishodia, Sethi et al., 2007). In addition, many studies

have verified this observation in several types of cancer including pancreatic cancer (D. W. Ahn, Seo et al., 2012; Lv, Song et al., 2008; Macha, Rachagani et al., 2013), esophageal (De Gottardi, Dumonceau et al., 2006; Guan, Hoque et al., 2014; Guan, Li et al., 2013; Yamada, Osawa et al., 2014), colon (An, Cheon et al., 2009; Cheon, Kim et al., 2006; J. M. Kim, Kang et al., 2010; Martinez-Becerra, Monte et al., 2012), breast (Jiang, Xiao et al., 2013; Noh, Chung et al., 2013), prostate (S. V. Singh, Zeng et al., 2005), hepatocellular (Moon, Park et al., 2011; J.-J. Shi, X.-L. Jia et al., 2015), as well as head and neck cancers (Macha, Matta et al., 2011b).

Apoptosis is also known to involve mitochondrial swelling, cavitation and other ultrastructural changes, suggesting that changes in mitochondrial morphology and function play important roles in the process (He, Xiao, Casiano, & Zhang, 2000). The mitochondria not only participate in caspase-dependent apoptosis but also significantly impact the Bcl-2 pathway during caspase-independent apoptosis. Mitochondrial alterations are one of the main pathways regulating Bcl family proteins and caspase-independent apoptosis. It is well-known that the balance of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins of the Bcl-2 family, play a role in regulating the effect of mitochondrial membrane permeability, mitochondrial function and Cyt-c release [19]; as two typical proteins of the Bcl family that restrain and promote apoptosis. Bax is known to antagonize Bcl-2 expression leading to an alteration of the mitochondrial membrane permeability followed by activation of caspases and apoptosis. Thus, we examined whether GS induces apoptosis by modulating the expression of Bcl-2 family members. Our current study results revealed that GS treatment induced alterations in mitochondrial pathway proteins through increasing the levels of truncated Bid (an activated form of Bid) and Bax at 48 h after treatment and decreasing the levels of Bcl-

2 and Bcl-xL at 48 h after treatment in a dose-dependent manner (Figure 18). Furthermore, GS treatment enhanced the Bax/Bcl-2 ratio in a dose-dependent manner. This was correlated with the significant loss of mitochondrial membrane potential observed in a dose-dependent manner, 48 h-post treatment (Figure 24-25). These findings were in accordance with the later reports in which treatment of GS was shown to downregulate the expression of antiapoptotic gene products including Bcl-2, Bcl-xL [24, 25]. Previous studies have shown that GS exerts no alterations in mitochondrial pathway protein Bax and Bcl-2 in normal cell lines (J. J. Shi, X. L. Jia et al., 2015). The mechanism is largely unknown; however, uncharacterized constituent(s) of GS may interact additively or synergistically to inhibit the viability of the cancer cells.

The degradation of extracellular matrix is an essential step in cancer invasion and metastasis [43]. A large number of literature studies shown that MMP-2 and MMP- 9 have been regarded as metastasis-related genes, which play important roles in cancer invasion and metastasis [43-48]. Studies have shown that extracellular matrix (ECM) degradation and neovascularization are the basis characteristic of tumor growth, invasion and metastasis [45, 46]. MMP-2 and MMP-9 could degrade the ECM and basement membrane collagen of blood vessels then promote tumor cell invasion and matastasis, while vascular endothelial growth by binding to VEGF receptor to promote angiogenesis, thus participating in the development and progression of tumors. In present study, we examined the expression and activities of MMP-2 and MMP-9 as well as VEGF in THP-1 cells. In the current study, our results revealed that GS treatment inhibited the expression of MMP-2 and MMP-9 in a dose-dependent manner (Figure 26). These findings were in accordance with the later reports in which treatment of GS was shown to downregulate the expression of VEGF in head and neck (Macha,

Matta et al., 2011a), colon (E. S. Kim, Hong et al., 2008), prostate (Xiao & Singh, 2008), and hepatocellular (J. J. Shi, X. L. Jia et al., 2015) cancers. Previous studies have shown that GS exerts no alterations in mitochondrial pathway protein Bax and Bcl-2 in normal cell lines (J. J. Shi, X. L. Jia et al., 2015). The mechanism is largely unknown; however, uncharacterized constituent(s) of GS may interact additively or synergistically to inhibit the viability of the cancer cells.

The inhibitor of apoptosis protein (IAP) family proteins reportedly block apoptosis due to their function as direct inhibitors that bind to and inhibit a number of caspases (Z. Gao, Tian et al., 2007). IAPs mainly inhibit caspases, inactivate apoptosis pathways (Gowda Saralamma, Lee et al., 2017). IAPs can inhibit the caspase-dependent apoptosis pathway by combining with caspases-3, −7 and −9; IAPs have become ideal target proteins for altering drug resistance of several key chemotherapeutic drugs. XIAP is the most potent caspase inhibitor in the IAP family of proteins, possessing three BIR domains at its N-terminal, which can regulate the death-receptor pathway and mitochondrial pathway-dependent apoptosis (26). BIR domain 3 in XIAP can effectively inhibit the activity of caspase-9 (27). The association between BIR1 and BIR2 can selectively inhibit caspases-3 and −7 (28). In the current study, we investigated GS's effects on the IAPs proteins, we examined the expression level of four members of the IAP family, xIAP, cIAP-1, and cIAP-2. The expression of the anti-apoptotic proteins; xIAP, cIAP-1, and cIAP-2 were significantly inhibited by guggulsterone in a dose-dependent manner at 48 h post-treatment (Figure 19). These findings were in accordance with the later reports in which treatment of GS was shown to downregulate the expression of antiapoptotic gene products including XIAP, survivin, ciap-1 and ciap-2 (An, Cheon et al., 2009; Leo, Therachiyil et al., 2019;

Shishodia, Sethi et al., 2007; S. V. Singh, S. Choi et al., 2007).

STAT3; an oncogenic transcription factor is a plausible therapeutic target for the treatment and prevention of leukemia, and is found constitutively active in several types of human neoplastic diseases in which contribute cancer progression and resistance to apoptosis. It has been demonstrated that cancer cells harboring anomalous STAT3 activity have elevated levels of anti-apoptotic proteins. Thus, cancer cells expressing constitutively activated STAT3 are more resistant to apoptosis (Zhang, Du, Liu, & Zhang, 2016). Over last decade, STAT3 has increasingly been noticed as a critical target for cancer therapy. A number of studies have introduced a variety of different STAT3 inhibitors and their mechanisms of action (Saini, Naidu et al., 2017). Inhibition of STAT3 results in deregulation of downstream target genes and consequently leads to growth inhibition and apoptosis (Siveen, Sikka et al., 2014). Natural compounds, like GS are not specific molecular inhibitors, which can be either a disadvantage or an advantage, depending on which other molecules are affected. One advantage of GS over oligonucleotide and peptidomimetic STAT3 inhibitors is that it is known to be safe and can be easily administered. GS treatment has been shown to block angiogenesis and metastasis by inactivation of STAT3 activity in several types of cancers (K. S. Ahn, Sethi et al., 2008; E. S. Kim, Hong et al., 2008). In the current study, we investigated the effect of guggulsterone on STAT3 activity using THP1 acute leukemia cells. GS was found to decrease phosphotyrosine, but not total STAT3 levels. Most natural compounds known to inhibit STAT3 only affect levels of phosphotyrosine STAT3, implying that the mechanism is indirect and involves either increases in activity of the physiological inhibitors of STAT3 (e.g. SOCS-1, SOCS-3, GRIM-19, PIAS and PTPRT) or decreases in signaling through upstream molecules (e.g. EGFR, Src, IL-6

receptor).

The major hurdle in leukemia treatment is probably related to adverse side effects of chemotherapy and resistance to chemotherapy drugs (Jamieson, Fox, Poi, & Strickland, 2016). One of the proposed methods of combating this issue is combination chemotherapy; which involves the administration of two or more drugs simultaneously which act differently against cancer cells (Al-Lazikani, Banerji et al. 2012). Combination therapy can provide benefits over monotherapy by improving the efficacy, decreasing the side effects and complications in patients, and hence, decreasing morbidity. However, most of the currently available combination therapy are hindered with high costs (Lu, Lu et al. 2013). Combining phytochemicals having anticancer potential with chemotherapeutic drug might provide a safe and cost-effective regimen for treating leukemia. Cisplatin is an effective cancer chemotherapy drug widely used for multiple types of tumor, including testicular, ovarian, lung, and head and neck cancer (Galanski, 2006). Although cisplatin has a strong therapeutic effect, drug resistance and serious side effects limit its clinical application (Reedijk, 2003). The combination of guggulsterone with cisplatin has previously been revealed to be effective against several malignancies (Bhat, Prabhu et al., 2017; Rebecca J. Leeman-Neill, Sarah E. Wheeler et al., 2009; Yue, Gao, Zou, Yu, & Zheng, 2017). However, there are no reports of this combination being investigated in leukemia either *in vitro* or *in vivo*. Therefore, in the present study, we investigated the anti-cancer effect of the combination of GS with the chemotherapeutic platinum-based agent cisplatin in leukemia cells and explored the mechanisms of their action in combination.

In the current study, we first assessed the effect of the combination treatment on cell viability using CCK-8 viability assay. Low doses of GS and Cis were combined in

the three leukemia cell lines. Combined action was determined based on combination indices (CI) and the dose response curves generated by CompuSyn software using Chou-Talalay method. Our results demonstrated that combination regimens with GS and Cis at subtoxic concentrations showed significant synergistic anticancer effects (CI <1) on AML (THP1 and U937) cells, however, no synergy was obtained in CML (K562) (Figure 28-35). In U937 cell line, stronger synergism was observed at lower concentrations than at higher concentrations. Whereas, THP1 showed greater synergism at higher concentrations (Figure 28-35). In contrary, antagonism was predominant in K562s cell lines, this can be explained by the gene expression signature of K562 human chronic myelogenous leukemia cells, which, unlike THP1 and U937 human acute leukemia cells, are relatively resistant to drug-induced apoptosis because, like the majority of human CML, they express the genotypic abnormality involving dysregulation of the p210 tyrosine kinase activity of the bcr-abl fusion oncoprotein ($p210^{bcr\text{-}abl}$) (Calabretta, 1991). The expression of $p210^{bcr\text{-}abl}$ in K562 cells is known to be responsible for their resistance to differentiation and drug-induced apoptosis (Bedi, Barber et al., 1995; Calabretta, 1991). Taken together, these findings suggested that GS might be used as a chemotherapeutic agent to enhance anticancer effects in acute leukemia cells when combined with Cis, which could decrease the dose of single medication. Thus, it can be useful to decrease the side effects of chemotherapy drugs, as it is effective to inhibit the proliferation of leukemia cells at a relatively low dose. Several studies have reported synergistic anti-tumor effects of GS in combination with conventional chemotherapeutic agents including erlotinib, cetuximab, bortezomib, and cisplatin in other types of cancers such as pancreatic, breast, liver, and gall bladder cancers (D. W. Ahn, Seo et al., 2012; Kong, He et al., 2015; Moon, Park et al., 2011;

H. B. Xu, Z. L. Shen, J. Fu, & L. Z. Xu, 2014; Yang, Lee et al., 2012).

After conducting the cytotoxicity assays of the combination treatment, we explored the mechanism of guggulsterone in potentiating the anti-tumor effect of cisplatin in K562s, THP-1 and U937 cells. Our flow cytometry results revealed that more cell apoptosis (71.24%) was induced in GS/Cis group than that by in the in GS alone group or Cis alone group (42.67% and 40.73%, respectively).

To further explain the mechanism of GS/Cis combination and to confirm apoptosis, we investigated the effect of GS/Cis combination on cell cycle distribution Interestingly, GS/Cis treatments induced cell cycle arrest in different phases which was dependent on the type of cells investigated (acute/chronic) and treatment dose (Figure 42-43). This suggests that cell cycle arrest can be a useful target for leukemia therapies. These differential sensitivity patterns seen within acute and chronic cell lines could be due to different cellular origin, genetic abnormalities and or expression of differential proteins by different types of cells. GS was previously showed to elicit minute level of cytotoxicity against normal cell lines (J. J. Shi, X. L. Jia et al., 2015; S. V. Singh, S. Choi et al., 2007). In addition, it was previously shown that GS treatment does not induce cell cycle arrest and apoptosis in normal cell lines, which may explain the reason why a cell line is more resistant to the growth inhibition by GS.

This study demonstrated that after GS/Cis treatment, the expression of caspase-9, caspase-3 and its endogenous substrate, cleavage of PARP, were enhanced. Taken together (Figure 38), these results suggested that the combination of GS and Cis induces apoptosis in leukemia cells. Meanwhile, at 48 h, the expression levels of truncated Bid (an activated form of Bid) and Bax increased, while the expression levels of Bcl-2 and Bcl-xL decreased (Figure 39). The overexpression of Bcl-2 and inhibition of Bax

expression are closely correlated with anti-apoptosis/apoptosis imbalance. This study demonstrated that GS/Cis combination treatment enhanced the Bax/Bcl-2 ratio was observed (Figure 39). This was correlated with the significant loss of mitochondrial membrane potential observed, 48 h-post treatment, which was higher than that observed after treatment with each drug individually. We also investigated GS's effects on the IAPs proteins, we examined the expression level of four members of the IAP family, xIAP and cIAP-1. The expression of the anti-apoptotic proteins; xIAP and cIAP-1 were significantly inhibited by GS/Cis combination compared to monotherapy (Figure 41). Collectively, showed that GS enhances the Cis-mediated apoptotic cell death as compared to GS or Cis alone in leukemia cells. To our knowledge, there are no previous reports on the combination of GS and Cis in leukemia cells. However, GS have been previously reported to enhance the effects of other chemotherapeutics such as gemcitabine (D. W. Ahn, Seo et al., 2012) and doxorubicin (Kong, He et al., 2015; H.-B. Xu, Z.-L. Shen et al., 2014; Xu, Li et al., 2011).

We analyzed the expression levels of MMPs which plays a vital role in in the invasion and metastasis of human malignancies (Brinckerhoff & Matrisian, 2002; Scherer, McIntyre, & Matrisian, 2008; X. F. Wang, Zhou et al., 2015). Among MMPs, MMP2 and MMP9 are known to be overexpressed and mediated higher rates of invasion and metastasis in various types of cancers [71–74]. In the current study, the GS/Cis combination inhibited the expression of MMP-2 and MMP-9 in THP1 leukemia cells (Figure 44), suggesting that the preferential cell growth inhibitory effect in the GS/Cis combination group might be at least partly attributed to the inhibition of MMPs which correlates with the higher rate of apoptosis induction by the GS/Cis combination. Taken together, our data revealed that GS/Cis combination could have the ability to

suppress the invasion and metastasis of human AML cells, thus probably effective in inhibiting the growth of AML cells.

We also investigated the effect of GS/Cis combination on STAT3 activity using THP1 acute leukemia cells. Our results revealed that GS/Cis combination suppressed phosphotyrosine and total STAT3 levels in THP1 leukemia cells, which provides the molecular basis of GS-mediated sensitization of leukemia cells to cisplatin. In this context, earlier reports also provide additional support for the combinational therapeutic potential of GS (Sarkhosh-Inanlou, Molaparast, Mohammadzadeh, & Shafiei-Irannejad, 2020; Sun, Lou et al., 2010), indicating that GS has the potential to affect the growth and proliferation of cancer cells alone and can sensitize malignant cells to cancer therapeutic drugs.

7. FUTURE DIRECTIONS

The rate of incidence of cancer and resulted deaths are alarming around the world despite the accessibility of various therapeutic options for cancer patients. Most modern medicines currently available for treating cancer are synthetic, mono-targeted, very expensive, less efficient and often possess severe side effects. Therefore, there is a critical need to develop alternative drugs for the management of cancer.

Phytochemicals, a family of naturally occurring compounds including polyphenols, carotenoids and steroids have been demonstrated to have anticancer activities against a variety of cancers both in vitro and in vivo. Among these compounds, guggulsterone (GS), a steroid by nature recently has attracted the attention of cancer researchers and

investigators for its anticancer potentials. GS has been shown to induce efficient apoptotic cell death in a variety of cancer cells. Interestingly, no apoptotic death was seen in healthy cells. A number of studies further showed significant cellular changes induced by GS via modulating distinct signaling molecules involved in carcinogen detoxification, cell proliferation, angiogenesis, metastasis, multi-drug resistance, etc. In addition, GS has been shown to sensitize the effects of chemotherapeutic drugs in in vitro system. These anticancer activities in preclinical settings are potentially beneficial in treating cancer.

Although in the present investigation we have demonstrated the in vitro anticancer potential of GS in leukemia cells, via targeting of a number of molecules associated with cell proliferation and growth and cancer stemness, further studies in appropriate animal models are essentially needed to confirm these results and for the potential future development of GS as a potent anticancer agent against leukemia. In addition, the mechanism of inhibition of proliferation in the chronic myelogenous leukemia need to be further investigated, given that apoptosis seems to not be the main mechanism involved.

Further studies directed towards target identification and pathway analysis could pave the way for the addition of GS to the management of anticancer therapy. Despite the availability of extensive pre- clinical data on anticancer potentials of GS, there is a lack of studies accounting for its safety and bioavailability, which needs to be pursued. Safety of long-term use of GS needs to be evaluated in clinical settings, but appears to be devoid of acute, subacute, chronic toxicity in rats, dogs, and monkeys; no mutagenic or teratogenic effects have been reported. Ayurvedic system of medicine describes GS as safe and efficient medicine; however, it should be used cautiously in combination

with prescribed drugs as it may modulate the activity of drug metabolizing enzymes. As soon as a consensus on its safety and bioavailability emerges, a planned Phase I clinical trials should be perused to validate its usefulness as anticancer agents and must be prioritized for different site- specific cancers. The outcome of these studies may lead to development of new and efficient therapeutic strategies for the management of cancer. Future work will focus on investigating the molecular mechanisms of GS on these cell lines to further the molecular targets of GS and hence identify reasons for the selective targeting toward lymphoid leukemia cell lines.

Since guggulsterone has shown optimistic anticancer activity against a variety of cancer cell lines including drug resistant cells, the activity and toxicity profile of the compounds could be carried out using suitable in vivo animal model study. Synergistic combinations could also be further evaluated in human xenograft model study in rodents. Proteomic studies need to be conducted to identify proteins to get the complete picture of their involvement in drug action. The proteins which have been found to be significantly expressed following drug treatments and associated with the antitumour activity of the compounds could be confirmed by western blot assay. Moreover, combination of protein expression profiling data with a network of protein-protein interactions and signalling pathways can be conducted to get a system level outlook of proteome changes associated with drug actions alone or in combinations.

8. CONCLUSION

Leukemia is a complex form of blood malignancy and considered is a major problem worldwide affecting many people each year. Unfortunately, current treatment options have several limitations and they usually have enormous side effects to the patients mainly related to high toxicity. Combination chemotherapy is now being preferred over single-drug treatment due to complexity of leukemia. But many such combinations increase the cost of overall treatment and side effects as well. Several epidemiological studies have shown that natural products are effective in the management of a number of human cancers and have the potentiality to be used against leukemia. Guggulsterone, a plant polyphenol has been found to possess cancer chemopreventive and therapeutic potential via inducing apoptotic cell death in a number of cancer cell lines. In this study, the anti-tumor effect of guggulsterone has been investigated alone, and in combination with the chemotherapeutic FDA approved drug cisplatin in leukemia cells. We demonstrated that guggulsterone inhibited the viability of human leukemia cells by inducing apoptosis through activation of the intrinsic mitochondrial pathway. Antitumour activity of guggusterone has been found to be associated with activation of caspase cascade, upregulation of the proapoptotic proteins (Bax and Bid) and downregulation of the antiapoptotic proteins (Bcl-2, Bcl-xL, xIAP, cIAP-1, cIAP-2 and survivin). Furthermore, guggulsterone was found to downregulate phosphotyrosine STAT3 but not total STAT3 levels. Another specific objective of this study was to exploit the anticancer potential of guggulsterone in combination with the existing chemotherapeutic approved platinum drug cisplatin. Our results revealed that guggulsterone acts synergistically with cisplatin in acute leukemia cell lines and

improves the chemosensitivity of cisplatin. Collectively, our results demonstrate that guggulsterone could serve as a potent natural anti-cancer agent that may be an effective treatment option for leukemia alone or in combination chemotherapy regimens. This may open a novel strategy to prevent or delay the development of platinum resistance and overall improve the treatment of leukemia.

9. REFERENCES

[1] Abdal Dayem, A., Choi, H. Y., et al. (2016). The anti-cancer effect of polyphenols against breast cancer and cancer stem cells: molecular mechanisms. *Nutrients, 8*(9), 581.

[2] Agarwal, R. C., Singh, S. P., et al. (1986). Clinical trial of gugulipid--a new hypolipidemic agent of plant origin in primary hyperlipidemia. *Indian J Med Res, 84*, 626-634.

[3] Aggarwal, B. B., & Shishodia, S. (2006). Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem Pharmacol, 71*(10), 1397-1421. doi:10.1016/j.bcp.2006.02.009

[4] Ahn, D. W., Seo, J. K., et al. (2012). Enhanced antitumor effect of combination therapy with gemcitabine and guggulsterone in pancreatic cancer. *Pancreas, 41*(7), 1048-1057. doi:10.1097/MPA.0b013e318249d62e

[5] Ahn, K. S., Sethi, G., et al. (2008). Guggulsterone, a farnesoid X receptor antagonist, inhibits constitutive and inducible STAT3 activation through induction of a protein tyrosine phosphatase SHP-1. *Cancer Res, 68*(11), 4406-4415. doi:10.1158/0008-5472.Can-07-6696

[6] Alegre, M. L., Frauwirth, K. A., et al. (2001). T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol, 1*(3), 220-228. doi:10.1038/35105024

[7] An, M. J., Cheon, J. H., et al. (2009). Guggulsterone induces apoptosis in colon cancer cells and inhibits tumor growth in murine colorectal cancer xenografts. *Cancer Lett, 279*(1), 93-100. doi:10.1016/j.canlet.2009.01.026

[8] Arem, H., & Loftfield, E. (2017). Cancer Epidemiology: A Survey of

Modifiable Risk Factors for Prevention and Survivorship. *American journal of lifestyle medicine, 12*(3), 200-210. doi:10.1177/1559827617700600

[9] Ashkenazi, A. (2008). Targeting the extrinsic apoptosis pathway in cancer. *Cytokine Growth Factor Rev, 19*(3-4), 325-331. doi:10.1016/j.cytogfr.2008.04.001

[10] Ashkenazi, A., & Dixit, V. M. (1998). Death receptors: signaling and modulation. *Science, 281*(5381), 1305-1308. doi:10.1126/science.281.5381.1305

[11] Attia, Y. M., Tawfiq, R. A., et al. (2017). The FXR Agonist, Obeticholic Acid, Suppresses HCC Proliferation & Metastasis: Role of IL-6/STAT3 Signalling Pathway. *Sci Rep, 7*(1), 12502. doi:10.1038/s41598-017-12629-4

[12] Badmus, J. A., Ekpo, O. E., et al. (2015). Antiproliferative and Apoptosis Induction Potential of the Methanolic Leaf Extract of Holarrhena floribunda (G. Don). *Evid Based Complement Alternat Med, 2015*, 756482. doi:10.1155/2015/756482

[13] Bailon-Moscoso, N., Romero-Benavides, J. C., et al. (2014). Development of anticancer drugs based on the hallmarks of tumor cells. *Tumour Biol, 35*(5), 3981-3995. doi:10.1007/s13277-014-1649-y

[14] Bedi, A., Barber, J. P., et al. (1995). BCR-ABL-mediated inhibition of apoptosis with delay of G2/M transition after DNA damage: a mechanism of resistance to multiple anticancer agents. *Blood, 86*(3), 1148-1158.

[15] Beg, M., Singhal, K. C., et al. (1996). A study of effect of guggulsterone on hyperlipidemia of secondary glomerulopathy. *Indian J Physiol Pharmacol, 40*(3), 237-240.

[16] Bendris, N., Lemmers, B., et al. (2015). Cell cycle, cytoskeleton dynamics and beyond: the many functions of cyclins and CDK inhibitors. *Cell Cycle, 14*(12), 1786-1798. doi:10.1080/15384101.2014.998085

[17] Bernasconi, N. L., Traggiai, E., et al. (2002). Maintenance of serological memory by polyclonal activation of human memory B cells. *Science, 298*(5601), 2199-2202. doi:10.1126/science.1076071

[18] Bhat, A. A., Prabhu, K. S., et al. (2017). Potential therapeutic targets of Guggulsterone in cancer. *Nutr Metab (Lond), 14*, 23-23. doi:10.1186/s12986-017-0180-8

[19] Boffetta, P., Couto, E., et al. (2010). Fruit and vegetable intake and overall cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC). *J Natl Cancer Inst, 102*(8), 529-537. doi:10.1093/jnci/djq072

[20] Brady, H. J. (2003). Apoptosis and leukaemia. *Br J Haematol, 123*(4), 577-585. doi:10.1046/j.1365-2141.2003.04663.x

[21] Bray, F., Ferlay, J., et al. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin, 68*(6), 394-424. doi:10.3322/caac.21492

[22] Brinckerhoff, C. E., & Matrisian, L. M. (2002). Matrix metalloproteinases: a tail of a frog that became a prince. *Nat Rev Mol Cell Biol, 3*(3), 207-214. doi:10.1038/nrm763

[23] Brusselmans, K., De Schrijver, E., et al. (2003). Epigallocatechin-3-gallate is a potent natural inhibitor of fatty acid synthase in intact cells and selectively induces apoptosis in prostate cancer cells. *Int J Cancer, 106*(6), 856-862. doi:10.1002/ijc.11317

[24] Buffler, P. A., Kwan, M. L., et al. (2005). Environmental and genetic risk factors for childhood leukemia: appraising the evidence. *Cancer Invest, 23*(1), 60-75.

[25] Burkhart, D. L., & Sage, J. (2008). Cellular mechanisms of tumour suppression

by the retinoblastoma gene. *Nat Rev Cancer, 8*(9), 671-682. doi:10.1038/nrc2399

[26] Calabretta, B. (1991). Inhibition of Protooncogene Expression by Antisense Oligodeoxynucleotides: Biological and Therapeutic Implications. *Cancer Res, 51*(17), 4505.

[27] Calame, K. L. (2001). Plasma cells: finding new light at the end of B cell development. *Nat Immunol, 2*(12), 1103-1108. doi:10.1038/ni1201-1103

[28] Cancer diagnosis and treatment statistics. (2015, 2015-07-31). Retrieved from https://www.cancerresearchuk.org/health-professional/cancer-statistics/diagnosis-and-treatment

[29] Cancer Facts & Figures 2012 | American Cancer Society. (2012). Retrieved from https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2012.html

[30] Centers for Disease Control and Prevention. (2018, Aug 16, 2018). Retrieved from https://www.cdc.gov/cancer/dcpc/research/articles/cancer_2020.htm

[31] Chambers, A. F., Groom, A. C., et al. (2002). Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer, 2*(8), 563-572. doi:10.1038/nrc865

[32] Chen, D., Milacic, V., et al. (2009). Metal complexes, their cellular targets and potential for cancer therapy. *Curr Pharm Des, 15*(7), 777-791. doi:10.2174/138161209787582183

[33] Chen, G. D., Qian, D. Y., et al. (2017). Down-regulation of p16 and MGMT promotes the anti-proliferative and pro-apoptotic effects of 5-Aza-dC and radiation on cervical cancer cells. *Cell Biochem Funct, 35*(8), 488-496. doi:10.1002/cbf.3282

[34] Cheon, J. H., Kim, J. S., et al. (2006). Plant sterol guggulsterone inhibits nuclear factor-kappaB signaling in intestinal epithelial cells by blocking IkappaB kinase and

ameliorates acute murine colitis. *Inflamm Bowel Dis, 12*(12), 1152-1161. doi:10.1097/01.mib.0000235830.94057.c6

[35] Chokkalingam, A. P., & Buffler, P. A. (2008). Genetic susceptibility to childhood leukaemia. *Radiation protection dosimetry, 132*(2), 119-129. doi:10.1093/rpd/ncn255

[36] Chou, T. C., & Martin, N. (2005). CompuSyn for drug combinations: PC software and user's guide: a computer program for quantitation of synergism and antagonism in drug combinations, and the determination of IC50 and ED50 and LD50 values. *ComboSyn, Paramus, NJ*.

[37] Choudhuri, R., Degraff, W., et al. (2011). Guggulsterone-mediated enhancement of radiosensitivity in human tumor cell lines. *Front Oncol, 1*, 19. doi:10.3389/fonc.2011.00019

[38] Cory, S., & Adams, J. M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer, 2*(9), 647-656. doi:10.1038/nrc883

[39] Cragg, G. M., & Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. *Biochim Biophys Acta, 1830*(6), 3670-3695. doi:10.1016/j.bbagen.2013.02.008

[40] Cui, J., Huang, L., et al. (2003). Guggulsterone is a farnesoid X receptor antagonist in coactivator association assays but acts to enhance transcription of bile salt export pump. *J Biol Chem, 278*(12), 10214-10220. doi:10.1074/jbc.M209323200

[41] Dai, Y., & Grant, S. (2003). Cyclin-dependent kinase inhibitors. *Curr Opin Pharmacol, 3*(4), 362-370.

[42] De Gottardi, A., Dumonceau, J. M., et al. (2006). Expression of the bile acid receptor FXR in Barrett's esophagus and enhancement of apoptosis by guggulsterone

in vitro. *Mol Cancer, 5*, 48. doi:10.1186/1476-4598-5-48

[43] Deng, R., Yang, D., et al. (2007). The hypolipidemic agent guggulsterone regulates the expression of human bile salt export pump: dominance of transactivation over farsenoid X receptor-mediated antagonism. *J Pharmacol Exp Ther, 320*(3), 1153-1162. doi:10.1124/jpet.106.113837

[44] Dertinger, S. D., Avlasevich, S. L., et al. (2014). Persistence of cisplatin-induced mutagenicity in hematopoietic stem cells: implications for secondary cancer risk following chemotherapy. *Toxicol Sci, 140*(2), 307-314. doi:10.1093/toxsci/kfu078

[45] Dev, S. (1999). Ancient-modern concordance in Ayurvedic plants: some examples. *Environ Health Perspect, 107*(10), 783-789. doi:10.1289/ehp.99107783

[46] Dimova, D. K., Stevaux, O., et al. (2003). Cell cycle-dependent and cell cycle-independent control of transcription by the Drosophila E2F/RB pathway. *Genes Dev, 17*(18), 2308-2320. doi:10.1101/gad.1116703

[47] Dixit, D., Ghildiyal, R., et al. (2013). Guggulsterone sensitizes glioblastoma cells to Sonic hedgehog inhibitor SANT-1 induced apoptosis in a Ras/NFκB dependent manner. *Cancer Lett, 336*(2), 347-358. doi:10.1016/j.canlet.2013.03.025

[48] Dohner, H., Weisdorf, D. J., et al. (2015). Acute Myeloid Leukemia. *N Engl J Med, 373*(12), 1136-1152. doi:10.1056/NEJMra1406184

[49] Dorai, T., & Aggarwal, B. B. (2004). Role of chemopreventive agents in cancer therapy. *Cancer Lett, 215*(2), 129-140. doi:10.1016/j.canlet.2004.07.013

[50] Du, C., Fang, M., et al. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell, 102*(1), 33-42. doi:10.1016/s0092-8674(00)00008-8

[51] Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol, 35*(4), 495-516. doi:10.1080/01926230701320337

[52] Enari, M., Sakahira, H., et al. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature, 391*(6662), 43-50. doi:10.1038/34112

[53] Fadeel, B., & Orrenius, S. (2005). Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J Intern Med, 258*(6), 479-517. doi:10.1111/j.1365-2796.2005.01570.x

[54] Farnsworth, N. R., Akerele, O., et al. (1985). Medicinal plants in therapy. *Bull World Health Organ, 63*(6), 965-981.

[55] Ferlay, J., Soerjomataram, I., et al. (2013). GLOBOCAN 2012 v1. 0, cancer incidence and mortality worldwide: IARC CancerBase No. 11. *Lyon, France: International agency for research on cancer, 2016*.

[56] Fischer, P. M., & Gianella-Borradori, A. (2003). CDK inhibitors in clinical development for the treatment of cancer. *Expert Opin Investig Drugs, 12*(6), 955-970. doi:10.1517/13543784.12.6.955

[57] Fürstenberg-Hägg, J., Zagrobelny, M., et al. (2013). Plant defense against insect herbivores. *Int J Mol Sci, 14*(5), 10242-10297. doi:10.3390/ijms140510242

[58] Gabrilovich, D. I., & Nagaraj, S. (2009). Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol, 9*(3), 162-174. doi:10.1038/nri2506

[59] Galanski, M. (2006). Recent developments in the field of anticancer platinum complexes. *Recent Pat Anticancer Drug Discov, 1*(2), 285-295. doi:10.2174/157489206777442287

[60] Gao, Y., Zeng, Y., et al. (2014). Retracted: Guggulsterone inhibits prostate cancer growth via inactivation of Akt regulated by ATP citrate lyase signaling. *Oncotarget*. doi:10.18632/oncotarget.2138

[61] Gao, Z., Tian, Y., et al. (2007). A dimeric SMAC/DIABLO peptide directly relieves caspase-3 inhibition by XIAP Dynamic and cooperative regulation of XIAP by SMAC/DIABLO. *Journal of Biological Chemistry, 282*(42), 30718-30727.

[62] Garrido, C., Galluzzi, L., et al. (2006). Mechanisms of cytochrome c release from mitochondria. *Cell Death Differ, 13*(9), 1423-1433. doi:10.1038/sj.cdd.4401950

[63] Giacinti, C., & Giordano, A. (2006). RB and cell cycle progression. *Oncogene, 25*(38), 5220-5227. doi:10.1038/sj.onc.1209615

[64] Gonzalez, V. M., Fuertes, M. A., et al. (2001). Is cisplatin-induced cell death always produced by apoptosis? *Mol Pharmacol, 59*(4), 657-663. doi:10.1124/mol.59.4.657

[65] Gowda Saralamma, V. V., Lee, H. J., et al. (2017). Inhibition of IAP's and activation of p53 leads to caspase-dependent apoptosis in gastric cancer cells treated with Scutellarein. *Oncotarget, 9*(5), 5993-6006. doi:10.18632/oncotarget.23202

[66] Greim, H., Kaden, D. A., et al. (2014). The bone marrow niche, stem cells, and leukemia: impact of drugs, chemicals, and the environment. *Annals of the New York Academy of Sciences, 1310*(1), 7-31. doi:10.1111/nyas.12362

[67] Gu, Y., Turck, C. W., et al. (1993). Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. *Nature, 366*(6456), 707-710. doi:10.1038/366707a0

[68] Guan, B., Hoque, A., et al. (2014). Amiloride and guggulsterone suppression of esophageal cancer cell growth in vitro and in nude mouse xenografts. *Front Biol*

(Beijing), 9(1), 75-81. doi:10.1007/s11515-014-1289-z

[69] Guan, B., Li, H., et al. (2013). Inhibition of farnesoid X receptor controls esophageal cancer cell growth in vitro and in nude mouse xenografts. *Cancer, 119*(7), 1321-1329. doi:10.1002/cncr.27910

[70] Guo, L., Huang, C., et al. (2017). Aberrant promoter hypermethylation of p16, survivin, and retinoblastoma in gastric cancer. *Bratisl Lek Listy, 118*(3), 164-168. doi:10.4149/bll_2017_033

[71] Hacker, G. (2000). The morphology of apoptosis. *Cell Tissue Res, 301*(1), 5-17. doi:10.1007/s004410000193

[72] Hafeez, B. B., Siddiqui, I. A., et al. (2008). A dietary anthocyanidin delphinidin induces apoptosis of human prostate cancer PC3 cells in vitro and in vivo: involvement of nuclear factor-kappaB signaling. *Cancer Res, 68*(20), 8564-8572. doi:10.1158/0008-5472.Can-08-2232

[73] Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. doi:10.1016/j.cell.2011.02.013

[74] Harakeh, S., Khan, I., et al. (2017). Role of nutrients and Phyto-compounds in the modulation of antimicrobial resistance. *Current drug metabolism, 18*(9), 858-867.

[75] Harbour, J. W., & Dean, D. C. (2000). Rb function in cell-cycle regulation and apoptosis. *Nat Cell Biol, 2*(4), E65-67. doi:10.1038/35008695

[76] He, J., Xiao, Y., et al. (2000). Role of mitochondrial cytochrome c in cocaine-induced apoptosis in coronary artery endothelial cells. *J Pharmacol Exp Ther, 295*(3), 896-903.

[77] Hill, M. M., Adrain, C., et al. (2004). Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. *The EMBO journal, 23*(10), 2134-

2145. doi:10.1038/sj.emboj.7600210

[78] Hofmann, W. K., de Vos, S., et al. (2001). Altered apoptosis pathways in mantle cell lymphoma detected by oligonucleotide microarray. *Blood, 98*(3), 787-794. doi:10.1182/blood.v98.3.787

[79] Hofseth, L. J., Hussain, S. P., et al. (2004). p53: 25 years after its discovery. *Trends Pharmacol Sci, 25*(4), 177-181. doi:10.1016/j.tips.2004.02.009

[80] Hui, A. M., Li, X., et al. (1999). Over-expression and lack of retinoblastoma protein are associated with tumor progression and metastasis in hepatocellular carcinoma. *Int J Cancer, 84*(6), 604-608. doi:10.1002/(sici)1097-0215(19991222)84:6<604::aid-ijc11>3.0.co;2-y

[81] Hussain, A. R., Al-Rasheed, M., et al. (2006). Curcumin induces apoptosis via inhibition of PI3'-kinase/AKT pathway in acute T cell leukemias. *Apoptosis : an international journal on programmed cell death, 11*(2), 245-254. doi:10.1007/s10495-006-3392-3

[82] Ichikawa, H., & Aggarwal, B. B. (2006). Guggulsterone inhibits osteoclastogenesis induced by receptor activator of nuclear factor-kappaB ligand and by tumor cells by suppressing nuclear factor-kappaB activation. *Clin Cancer Res, 12*(2), 662-668. doi:10.1158/1078-0432.Ccr-05-1749

[83] Ishikawa, M., Nakayama, K., et al. (2014). Therapy-related myelodysplastic syndrome and acute myeloid leukemia following chemotherapy (paclitaxel and carboplatin) and radiation therapy in ovarian cancer: a case report. *Eur J Gynaecol Oncol, 35*(4), 443-448.

[84] Jacks, T., Fazeli, A., et al. (1992). Effects of an Rb mutation in the mouse. *Nature, 359*(6393), 295-300. doi:10.1038/359295a0

[85] Jamieson, G. C., Fox, J. A., et al. (2016). Molecular and Pharmacologic Properties of the Anticancer Quinolone Derivative Vosaroxin: A New Therapeutic Agent for Acute Myeloid Leukemia. *Drugs, 76*(13), 1245-1255. doi:10.1007/s40265-016-0614-z

[86] Jiang, G., Xiao, X., et al. (2013). Targeting beta-catenin signaling to induce apoptosis in human breast cancer cells by z-guggulsterone and Gugulipid extract of Ayurvedic medicine plant Commiphora mukul. *BMC Complement Altern Med, 13*, 203. doi:10.1186/1472-6882-13-203

[87] Joza, N., Susin, S. A., et al. (2001). Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature, 410*(6828), 549-554. doi:10.1038/35069004

[88] Kaatsch, P. (2010). Epidemiology of childhood cancer. *Cancer Treatment Reviews, 36*(4), 277-285. doi:10.1016/j.ctrv.2010.02.003

[89] Kandikattu, H. K., Rachitha, P., et al. (2017). Anti-inflammatory and anti-oxidant effects of Cardamom (Elettaria repens (Sonn.) Baill) and its phytochemical analysis by 4D GCXGC TOF-MS. *Biomedicine & Pharmacotherapy, 91*, 191-201.

[90] Kantarjian, H. M., O'Brien, S., et al. (2002). Imatinib mesylate therapy for relapse after allogeneic stem cell transplantation for chronic myelogenous leukemia. *Blood, 100*(5), 1590-1595.

[91] Kapoor, S. (2008). Guggulsterone: a potent farnesoid X receptor antagonist and its rapidly evolving role as a systemic anticarcinogenic agent. *Hepatology, 48*(6), 2090-2091. doi:10.1002/hep.22601

[92] Key, T. J. (2011). Fruit and vegetables and cancer risk. *Br J Cancer, 104*(1), 6-11. doi:10.1038/sj.bjc.6606032

[93] Khan, M. T., Wagner, L., 2nd, et al. (2006). Akt kinase phosphorylation of inositol 1,4,5-trisphosphate receptors. *J Biol Chem, 281*(6), 3731-3737. doi:10.1074/jbc.M509262200

[94] Kim, B. H., Yoon, J. H., et al. (2013). Guggulsterone attenuates activation and survival of hepatic stellate cell by inhibiting nuclear factor kappa B activation and inducing apoptosis. *J Gastroenterol Hepatol, 28*(12), 1859-1868. doi:10.1111/jgh.12314

[95] Kim, E. S., Hong, S. Y., et al. (2008). Guggulsterone inhibits angiogenesis by blocking STAT3 and VEGF expression in colon cancer cells. *Oncol Rep, 20*(6), 1321-1327.

[96] Kim, J. M., Kang, H. W., et al. (2010). Novel guggulsterone derivative GG-52 inhibits NF-kappaB signaling in intestinal epithelial cells and attenuates acute murine colitis. *Lab Invest, 90*(7), 1004-1015. doi:10.1038/labinvest.2010.54

[97] Kischkel, F. C., Lawrence, D. A., et al. (2000). Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity, 12*(6), 611-620. doi:10.1016/s1074-7613(00)80212-5

[98] Kondo, M. (2010). Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *Immunological reviews, 238*(1), 37-46. doi:10.1111/j.1600-065X.2010.00963.x

[99] Kondo, M., Wagers, A. J., et al. (2003). Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol, 21*, 759-806. doi:10.1146/annurev.immunol.21.120601.141007

[100] Kong, J. N., He, Q., et al. (2015). Guggulsterone and bexarotene induce secretion of exosome-associated breast cancer resistance protein and reduce

doxorubicin resistance in MDA-MB-231 cells. *Int J Cancer, 137*(7), 1610-1620. doi:10.1002/ijc.29542

[101] Kostova, I. (2006). Platinum complexes as anticancer agents. *Recent Pat Anticancer Drug Discov, 1*(1), 1-22. doi:10.2174/157489206775246458

[102] Krishnamurthy, K., Wang, G., et al. (2008). Deoxycholate promotes survival of breast cancer cells by reducing the level of pro-apoptotic ceramide. *Breast Cancer Res, 10*(6), R106. doi:10.1186/bcr2211

[103] Krug, U., Ganser, A., et al. (2002). Tumor suppressor genes in normal and malignant hematopoiesis. *Oncogene, 21*(21), 3475-3495. doi:10.1038/sj.onc.1205322

[104] Lacour, B., Guyot-Goubin, A., et al. (2010). Incidence of childhood cancer in France: National Children Cancer Registries, 2000-2004. *Eur J Cancer Prev, 19*(3), 173-181.

[105] Larsen, B. D., Rampalli, S., et al. (2010). Caspase 3/caspase-activated DNase promote cell differentiation by inducing DNA strand breaks. *Proc Natl Acad Sci U S A, 107*(9), 4230-4235. doi:10.1073/pnas.0913089107

[106] Lazebnik, Y. A., Kaufmann, S. H., et al. (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature, 371*(6495), 346-347. doi:10.1038/371346a0

[107] Lee, M. H., Reynisdottir, I., et al. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev, 9*(6), 639-649. doi:10.1101/gad.9.6.639

[108] Leeman-Neill, R. J., Wheeler, S. E., et al. (2009). Guggulsterone enhances head and neck cancer therapies via inhibition of signal transducer and activator of transcription-3. *Carcinogenesis, 30*(11), 1848-1856. doi:10.1093/carcin/bgp211

[109] Leeman-Neill, R. J., Wheeler, S. E., et al. (2009). Guggulsterone enhances head and neck cancer therapies via inhibition of signal transducer and activator of transcription-3. *Carcinogenesis, 30*(11), 1848-1856. doi:10.1093/carcin/bgp211

[110] Leist, M., Single, B., et al. (1997). Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med, 185*(8), 1481-1486. doi:10.1084/jem.185.8.1481

[111] Leo, R., Therachiyil, L., et al. (2019). Protein Expression Profiling Identifies Key Proteins and Pathways Involved in Growth Inhibitory Effects Exerted by Guggulsterone in Human Colorectal Cancer Cells. *Cancers (Basel), 11*(10). doi:10.3390/cancers11101478

[112] Lessene, G., Czabotar, P. E., et al. (2008). BCL-2 family antagonists for cancer therapy. *Nat Rev Drug Discov, 7*(12), 989-1000. doi:10.1038/nrd2658

[113] Leukaemia (all subtypes combined) statistics. (2015, 2015-05-14). Retrieved from https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/leukaemia

[114] Li, C., Zang, Y., et al. (2009). Bortezomib up-regulates activated signal transducer and activator of transcription-3 and synergizes with inhibitors of signal transducer and activator of transcription-3 to promote head and neck squamous cell carcinoma cell death. *Mol Cancer Ther, 8*(8), 2211-2220. doi:10.1158/1535-7163.Mct-09-0327

[115] Li, P., Nijhawan, D., et al. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell, 91*(4), 479-489. doi:10.1016/s0092-8674(00)80434-1

[116] Li, X., Huang, T., et al. (2013). Synergistic apoptotic effect of crocin and

cisplatin on osteosarcoma cells via caspase induced apoptosis. *Toxicology letters, 221*(3), 197-204.

[117] Li, X., Marani, M., et al. (2001). Adenovirus-mediated Bax overexpression for the induction of therapeutic apoptosis in prostate cancer. *Cancer Res, 61*(1), 186-191.

[118] Lim, S., & Kaldis, P. (2013). Cdks, cyclins and CKIs: roles beyond cell cycle regulation. *Development, 140*(15), 3079-3093. doi:10.1242/dev.091744

[119] Liu, Z., Zhai, J., et al. (2016). Assessment of anti-diabetic activity of the aqueous extract of leaves of Astilboides tabularis. *Journal of ethnopharmacology, 194*, 635-641.

[120] Locksley, R. M., Killeen, N., et al. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell, 104*(4), 487-501.

[121] Loeb, L. A., Springgate, C. F., et al. (1974). Errors in DNA replication as a basis of malignant changes. *Cancer Res, 34*(9), 2311-2321.

[122] Lv, N., Song, M. Y., et al. (2008). Guggulsterone, a plant sterol, inhibits NF-kappaB activation and protects pancreatic beta cells from cytokine toxicity. *Mol Cell Endocrinol, 289*(1-2), 49-59. doi:10.1016/j.mce.2008.02.001

[123] Macha, M. A., Matta, A., et al. (2010). 14-3-3 zeta is a molecular target in guggulsterone induced apoptosis in head and neck cancer cells. *BMC Cancer, 10*, 655. doi:10.1186/1471-2407-10-655

[124] Macha, M. A., Matta, A., et al. (2011a). Guggulsterone (GS) inhibits smokeless tobacco and nicotine-induced NF-kappaB and STAT3 pathways in head and neck cancer cells. *Carcinogenesis, 32*(3), 368-380. doi:10.1093/carcin/bgq278

[125] Macha, M. A., Matta, A., et al. (2011b). Guggulsterone targets smokeless tobacco induced PI3K/Akt pathway in head and neck cancer cells. *PLoS One, 6*(2),

e14728. doi:10.1371/journal.pone.0014728

[126] Macha, M. A., Rachagani, S., et al. (2013). Guggulsterone decreases proliferation and metastatic behavior of pancreatic cancer cells by modulating JAK/STAT and Src/FAK signaling. *Cancer Lett, 341*(2), 166-177. doi:10.1016/j.canlet.2013.07.037

[127] Mann, J. (2002). Natural products in cancer chemotherapy: past, present and future. *Nat Rev Cancer, 2*(2), 143-148. doi:10.1038/nrc723

[128] Martin, K. R. (2006). Targeting apoptosis with dietary bioactive agents. *Exp Biol Med (Maywood), 231*(2), 117-129. doi:10.1177/153537020623100201

[129] Martinez-Becerra, P., Monte, I., et al. (2012). Up-regulation of FXR isoforms is not required for stimulation of the expression of genes involved in the lack of response of colon cancer to chemotherapy. *Pharmacol Res, 66*(5), 419-427. doi:10.1016/j.phrs.2012.07.005

[130] Mathers, C. D., Shibuya, K., et al. (2002). Global and regional estimates of cancer mortality and incidence by site: I. Application of regional cancer survival model to estimate cancer mortality distribution by site. *BMC Cancer, 2*, 36.

[131] Matsuoka, S., Edwards, M. C., et al. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev, 9*(6), 650-662. doi:10.1101/gad.9.6.650

[132] Mbiantcha, M., Almas, J., et al. (2018). Analgesic, anti-inflammatory and anticancer activities of Combretin A and Combretin B isolated from Combretum fragrans F. HOFFM (Combretaceae) leaves. *Inflammopharmacology, 26*(6), 1429-1440.

[133] Meeran, S. M., & Katiyar, S. K. (2008). Cell cycle control as a basis for cancer

chemoprevention through dietary agents. *Front Biosci, 13*, 2191-2202. doi:10.2741/2834

[134] Mehrotra, M., Williams, C. R., et al. (2013). Hematopoietic stem cells give rise to osteo-chondrogenic cells. *Blood cells, molecules & diseases, 50*(1), 41-49. doi:10.1016/j.bcmd.2012.08.003

[135] Meirow, D., & Nugent, D. (2001). The effects of radiotherapy and chemotherapy on female reproduction. *Hum Reprod Update, 7*(6), 535-543. doi:10.1093/humupd/7.6.535

[136] Milacic, V., Fregona, D., et al. (2008). Gold complexes as prospective metal-based anticancer drugs. *Histol Histopathol, 23*(1), 101-108. doi:10.14670/hh-23.101

[137] Moon, D. O., Park, S. Y., et al. (2011). Guggulsterone sensitizes hepatoma cells to TRAIL-induced apoptosis through the induction of CHOP-dependent DR5: involvement of ROS-dependent ER-stress. *Biochem Pharmacol, 82*(11), 1641-1650. doi:10.1016/j.bcp.2011.08.019

[138] Neuhouser, M. L., Thompson, B., et al. (2004). Higher fat intake and lower fruit and vegetables intakes are associated with greater acculturation among Mexicans living in Washington State. *J Am Diet Assoc, 104*(1), 51-57. doi:10.1016/j.jada.2003.10.015

[139] Newman, D. J., & Cragg, G. M. (2007). Natural products as sources of new drugs over the last 25 years. *J Nat Prod, 70*(3), 461-477. doi:10.1021/np068054v

[140] Nityanand, S., Srivastava, J. S., et al. (1989). Clinical trials with gugulipid. A new hypolipidaemic agent. *J Assoc Physicians India, 37*(5), 323-328.

[141] Noh, E. M., Chung, E. Y., et al. (2013). Cis-guggulsterone inhibits the IKK/NF-κB pathway, whereas trans-guggulsterone inhibits MAPK/AP-1 in MCF-7 breast

cancer cells: guggulsterone regulates MMP-9 expression in an isomer-specific manner. *Int J Mol Med, 31*(2), 393-399. doi:10.3892/ijmm.2012.1214

[142] Norbury, C. J., & Hickson, I. D. (2001). Cellular responses to DNA damage. *Annu Rev Pharmacol Toxicol, 41*, 367-401. doi:10.1146/annurev.pharmtox.41.1.367

[143] Nurse, P. (2000). A long twentieth century of the cell cycle and beyond. *Cell, 100*(1), 71-78. doi:10.1016/s0092-8674(00)81684-0

[144] Øverby, A., Zhao, C.-M., et al. (2014). Plant phytochemicals: Potential anticancer agents against gastric cancer. *Curr Opin Pharmacol, 19*, 6-10.

[145] Owsley, E., & Chiang, J. Y. (2003). Guggulsterone antagonizes farnesoid X receptor induction of bile salt export pump but activates pregnane X receptor to inhibit cholesterol 7alpha-hydroxylase gene. *Biochem Biophys Res Commun, 304*(1), 191-195. doi:10.1016/s0006-291x(03)00551-5

[146] Paschka, A. G., Butler, R., et al. (1998). Induction of apoptosis in prostate cancer cell lines by the green tea component, (-)-epigallocatechin-3-gallate. *Cancer Lett, 130*(1-2), 1-7. doi:10.1016/s0304-3835(98)00084-6

[147] Pavia, M., Pileggi, C., et al. (2006). Association between fruit and vegetable consumption and oral cancer: a meta-analysis of observational studies. *Am J Clin Nutr, 83*(5), 1126-1134. doi:10.1093/ajcn/83.5.1126

[148] Peng, Z., Raufman, J. P., et al. (2012). Src-mediated cross-talk between farnesoid X and epidermal growth factor receptors inhibits human intestinal cell proliferation and tumorigenesis. *PLoS One, 7*(10), e48461. doi:10.1371/journal.pone.0048461

[149] Perez, R. P. (1998). Cellular and molecular determinants of cisplatin resistance. *Eur J Cancer, 34*(10), 1535-1542. doi:10.1016/s0959-8049(98)00227-5

[150] Pestell, K. E., Hobbs, S. M., et al. (2000). Effect of p53 status on sensitivity to platinum complexes in a human ovarian cancer cell line. *Mol Pharmacol, 57*(3), 503-511. doi:10.1124/mol.57.3.503

[151] Pietras, K., & Ostman, A. (2010). Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res, 316*(8), 1324-1331. doi:10.1016/j.yexcr.2010.02.045

[152] Prabhu, K. S., Siveen, K. S., et al. (2017). Targeting of X-linked inhibitor of apoptosis protein and PI3-kinase/AKT signaling by embelin suppresses growth of leukemic cells. *PLoS One, 12*(7), e0180895. doi:10.1371/journal.pone.0180895

[153] Qian, Y., Luckey, C., et al. (1992). Biological function of the retinoblastoma protein requires distinct domains for hyperphosphorylation and transcription factor binding. *Mol Cell Biol, 12*(12), 5363-5372. doi:10.1128/mcb.12.12.5363

[154] Redd, W. H., Montgomery, G. H., et al. (2001). Behavioral intervention for cancer treatment side effects. *J Natl Cancer Inst, 93*(11), 810-823. doi:10.1093/jnci/93.11.810

[155] Reedijk, J. (2003). New clues for platinum antitumor chemistry: kinetically controlled metal binding to DNA. *Proc Natl Acad Sci U S A, 100*(7), 3611-3616. doi:10.1073/pnas.0737293100

[156] Ren, Y., & Savill, J. (1998). Apoptosis: the importance of being eaten. *Cell Death Differ, 5*(7), 563-568. doi:10.1038/sj.cdd.4400407

[157] Rodriguez-Abreu, D., Bordoni, A., et al. (2007). Epidemiology of hematological malignancies. *Ann Oncol, 18 Suppl 1*, i3-i8. doi:10.1093/annonc/mdl443

[158] Saelens, X., Festjens, N., et al. (2004). Toxic proteins released from mitochondria in cell death. *Oncogene, 23*(16), 2861-2874.

doi:10.1038/sj.onc.1207523

[159] Saini, U., Naidu, S., et al. (2017). Elevated STAT3 expression in ovarian cancer ascites promotes invasion and metastasis: a potential therapeutic target. *Oncogene, 36*(2), 168-181. doi:10.1038/onc.2016.197

[160] Samudio, I., Konopleva, M., et al. (2005). Guggulsterones induce apoptosis and differentiation in acute myeloid leukemia: identification of isomer-specific antileukemic activities of the pregnadienedione structure. *Mol Cancer Ther, 4*(12), 1982-1992. doi:10.1158/1535-7163.Mct-05-0247

[161] Saraste, A., & Pulkki, K. (2000). Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res, 45*(3), 528-537. doi:10.1016/s0008-6363(99)00384-3

[162] Sarkhosh-Inanlou, R., Molaparast, M., et al. (2020). Sanguinarine enhances cisplatin sensitivity via glutathione depletion in cisplatin-resistant ovarian cancer (A2780) cells. *Chemical Biology & Drug Design, 95*(2), 215-223.

[163] Satyavati, G. V. (1988). Gum guggul (Commiphora mukul)--the success story of an ancient insight leading to a modern discovery. *Indian J Med Res, 87*, 327-335.

[164] Satyavati, G. V., Dwarakanath, C., et al. (1969). Experimental studies on the hypocholesterolemic effect of Commiphora mukul. Engl. (Guggul). *Indian J Med Res, 57*(10), 1950-1962.

[165] Scherer, R. L., McIntyre, J. O., et al. (2008). Imaging matrix metalloproteinases in cancer. *Cancer Metastasis Rev, 27*(4), 679-690. doi:10.1007/s10555-008-9152-9

[166] Segal-Bendirdjian, E., & Jacquemin-Sablon, A. (1995). Cisplatin resistance in a murine leukemia cell line is associated with a defective apoptotic process. *Exp Cell Res, 218*(1), 201-212. doi:10.1006/excr.1995.1148

[167] Seiter, K., Katragadda, S., et al. (2009). Temozolomide and cisplatin in

relapsed/refractory acute leukemia. *J Hematol Oncol, 2*, 21. doi:10.1186/1756-8722-2-21

[168] Shariat, S. F., Tokunaga, H., et al. (2004). p53, p21, pRB, and p16 expression predict clinical outcome in cystectomy with bladder cancer. *J Clin Oncol, 22*(6), 1014-1024. doi:10.1200/jco.2004.03.118

[169] Shi, J.-J., Jia, X.-L., et al. (2015). Guggulsterone induces apoptosis of human hepatocellular carcinoma cells through intrinsic mitochondrial pathway. *World J Gastroenterol, 21*(47), 13277-13287. doi:10.3748/wjg.v21.i47.13277

[170] Shi, J. J., Jia, X. L., et al. (2015). Guggulsterone induces apoptosis of human hepatocellular carcinoma cells through intrinsic mitochondrial pathway. *World J Gastroenterol, 21*(47), 13277-13287. doi:10.3748/wjg.v21.i47.13277

[171] Shishodia, S., & Aggarwal, B. B. (2004). Guggulsterone inhibits NF-kappaB and IkappaBalpha kinase activation, suppresses expression of anti-apoptotic gene products, and enhances apoptosis. *J Biol Chem, 279*(45), 47148-47158. doi:10.1074/jbc.M408093200

[172] Shishodia, S., Sethi, G., et al. (2007). Guggulsterone inhibits tumor cell proliferation, induces S-phase arrest, and promotes apoptosis through activation of c-Jun N-terminal kinase, suppression of Akt pathway, and downregulation of antiapoptotic gene products. *Biochem Pharmacol, 74*(1), 118-130. doi:10.1016/j.bcp.2007.03.026

[173] Siegel, R. L., Miller, K. D., et al. (2017). Cancer Statistics, 2017. *CA Cancer J Clin, 67*(1), 7-30. doi:10.3322/caac.21387

[174] Siegel, R. L., Miller, K. D., et al. (2019). Cancer statistics, 2019. *CA Cancer J Clin, 69*(1), 7-34. doi:10.3322/caac.21551

[175] Sinal, C. J., & Gonzalez, F. J. (2002). Guggulsterone: an old approach to a new problem. *Trends Endocrinol Metab, 13*(7), 275-276.

[176] Singh, R. B., Niaz, M. A., et al. (1994). Hypolipidemic and antioxidant effects of Commiphora mukul as an adjunct to dietary therapy in patients with hypercholesterolemia. *Cardiovasc Drugs Ther, 8*(4), 659-664. doi:10.1007/bf00877420

[177] Singh, S. V., Choi, S., et al. (2007). Guggulsterone-induced apoptosis in human prostate cancer cells is caused by reactive oxygen intermediate dependent activation of c-Jun NH2-terminal kinase. *Cancer Res, 67*(15), 7439-7449. doi:10.1158/0008-5472.CAN-07-0120

[178] Singh, S. V., Choi, S., et al. (2007). Guggulsterone-induced apoptosis in human prostate cancer cells is caused by reactive oxygen intermediate dependent activation of c-Jun NH2-terminal kinase. *Cancer Res, 67*(15), 7439-7449. doi:10.1158/0008-5472.Can-07-0120

[179] Singh, S. V., Zeng, Y., et al. (2005). Caspase-dependent apoptosis induction by guggulsterone, a constituent of Ayurvedic medicinal plant Commiphora mukul, in PC-3 human prostate cancer cells is mediated by Bax and Bak. *Mol Cancer Ther, 4*(11), 1747-1754. doi:10.1158/1535-7163.Mct-05-0223

[180] Siveen, K. S., Mustafa, N., et al. (2014). Thymoquinone overcomes chemoresistance and enhances the anticancer effects of bortezomib through abrogation of NF-κB regulated gene products in multiple myeloma xenograft mouse model. *Oncotarget, 5*(3), 634-648. doi:10.18632/oncotarget.1596

[181] Siveen, K. S., Sikka, S., et al. (2014). Targeting the STAT3 signaling pathway in cancer: role of synthetic and natural inhibitors. *Biochim Biophys Acta, 1845*(2),

136-154. doi:10.1016/j.bbcan.2013.12.005

[182] Stevaux, O., & Dyson, N. J. (2002). A revised picture of the E2F transcriptional network and RB function. *Curr Opin Cell Biol, 14*(6), 684-691. doi:10.1016/s0955-0674(02)00388-5

[183] Stewart, B. W., & Kleihues, P. (2003). World cancer report.

[184] Sun, M., Lou, W., et al. (2010). Sanguinarine suppresses prostate tumor growth and inhibits survivin expression. *Genes & cancer, 1*(3), 283-292.

[185] Swerdlow, S. H., International Agency for Research on, C., et al. (2008). *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*: International Agency for Research on Cancer.

[186] Szapary, P. O., Wolfe, M. L., et al. (2003). Guggulipid for the treatment of hypercholesterolemia: a randomized controlled trial. *Jama, 290*(6), 765-772. doi:10.1001/jama.290.6.765

[187] Tang, J. L., Yeh, S. H., et al. (1992). Inactivation of the retinoblastoma gene in acute myelogenous leukaemia. *Br J Haematol, 82*(3), 502-507. doi:10.1111/j.1365-2141.1992.tb06459.x

[188] Tanida, S., Mizoshita, T., et al. (2012). Mechanisms of Cisplatin-Induced Apoptosis and of Cisplatin Sensitivity: Potential of BIN1 to Act as a Potent Predictor of Cisplatin Sensitivity in Gastric Cancer Treatment. *International journal of surgical oncology, 2012*, 862879-862879. doi:10.1155/2012/862879

[189] Thornberry, N. A. (1998). Caspases: key mediators of apoptosis. *Chemistry & biology, 5*(5), R97-R103. doi:10.1016/s1074-5521(98)90615-9

[190] Toyoshima, H., & Hunter, T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell, 78*(1), 67-74. doi:10.1016/0092-

8674(94)90573-8

[191] Travis, L. B., Andersson, M., et al. (2000). Treatment-associated leukemia following testicular cancer. *J Natl Cancer Inst, 92*(14), 1165-1171. doi:10.1093/jnci/92.14.1165

[192] Urizar, N. L., Liverman, A. B., et al. (2002). A natural product that lowers cholesterol as an antagonist ligand for FXR. *Science, 296*(5573), 1703-1706. doi:10.1126/science.1072891

[193] Urizar, N. L., & Moore, D. D. (2003). GUGULIPID: a natural cholesterol-lowering agent. *Annu Rev Nutr, 23*, 303-313. doi:10.1146/annurev.nutr.23.011702.073102

[194] Vallmanya Llena, F. R., Laborda Rodriguez, A., et al. (2006). [Immunohistochemical expression of p53, p21, p16, and cyclin D1 in superficial bladder cancer. A tissue microarray study]. *Actas Urol Esp, 30*(8), 754-762. doi:10.1016/s0210-4806(06)73532-x

[195] van Loo, G., van Gurp, M., et al. (2002). The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. *Cell Death Differ, 9*(1), 20-26. doi:10.1038/sj.cdd.4400970

[196] Vermeulen, K., Van Bockstaele, D. R., et al. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif, 36*(3), 131-149. doi:10.1046/j.1365-2184.2003.00266.x

[197] Wang, F. L., Yang, Y., et al. (2017). Correlation between methylation of the p16 promoter and cervical cancer incidence. *Eur Rev Med Pharmacol Sci, 21*(10), 2351-2356.

[198] Wang, H., Naghavi, M., et al. (2016). Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. *The lancet, 388*(10053), 1459-1544.

[199] Wang, W. C., Uen, Y. H., et al. (2012). Protective effect of guggulsterone against cardiomyocyte injury induced by doxorubicin in vitro. *BMC Complement Altern Med, 12*, 138. doi:10.1186/1472-6882-12-138

[200] Wang, X. F., Zhou, Q. M., et al. (2015). Glycyrrhetinic acid potently suppresses breast cancer invasion and metastasis by impairing the p38 MAPK-AP1 signaling axis. *Expert Opin Ther Targets, 19*(5), 577-587. doi:10.1517/14728222.2015.1012156

[201] Weinberg, R. A. (2013). *The Biology of Cancer: Second International Student Edition*: Garland Science.

[202] Weissman, I. L. (2000). Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science, 287*(5457), 1442-1446. doi:10.1126/science.287.5457.1442

[203] Wikenheiser-Brokamp, K. A. (2006). Retinoblastoma regulatory pathway in lung cancer. *Curr Mol Med, 6*(7), 783-793.

[204] Wong, E., & Giandomenico, C. M. (1999). Current status of platinum-based antitumor drugs. *Chem Rev, 99*(9), 2451-2466. doi:10.1021/cr980420v

[205] Wu, J., Xia, C., et al. (2002). The hypolipidemic natural product guggulsterone acts as an antagonist of the bile acid receptor. *Mol Endocrinol, 16*(7), 1590-1597. doi:10.1210/mend.16.7.0894

[206] Xiao, D., & Singh, S. V. (2008). z-Guggulsterone, a constituent of Ayurvedic

medicinal plant Commiphora mukul, inhibits angiogenesis in vitro and in vivo. *Mol Cancer Ther, 7*(1), 171-180. doi:10.1158/1535-7163.Mct-07-0491

[207] Xiao, D., Zeng, Y., et al. (2011). Reactive oxygen species-dependent apoptosis by gugulipid extract of Ayurvedic medicine plant Commiphora mukul in human prostate cancer cells is regulated by c-Jun N-terminal kinase. *Mol Pharmacol, 79*(3), 499-507. doi:10.1124/mol.110.068551

[208] Xiong, Y., Hannon, G. J., et al. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature, 366*(6456), 701-704. doi:10.1038/366701a0

[209] Xu, H.-B., Shen, Z.-L., et al. (2014). Reversal of doxorubicin resistance by guggulsterone of Commiphora mukul in vivo. *Phytomedicine, 21*(11), 1221-1229. doi:10.1016/j.phymed.2014.06.003

[210] Xu, H. B., Li, L., et al. (2011). Reversal of multidrug resistance by guggulsterone in drug-resistant MCF-7 cell lines. *Chemotherapy, 57*(1), 62-70. doi:10.1159/000321484

[211] Xu, H. B., Shen, Z. L., et al. (2014). Reversal of doxorubicin resistance by guggulsterone of Commiphora mukul in vivo. *Phytomedicine, 21*(11), 1221-1229. doi:10.1016/j.phymed.2014.06.003

[212] Yamada, T., Osawa, S., et al. (2010). Guggulsterone suppresses bile acid-induced and constitutive caudal-related homeobox 2 expression in gut-derived adenocarcinoma cells. *Anticancer Res, 30*(6), 1953-1960.

[213] Yamada, T., Osawa, S., et al. (2014). Guggulsterone, a plant-derived inhibitor of NF-TB, suppresses CDX2 and COX-2 expression and reduces the viability of esophageal adenocarcinoma cells. *Digestion, 90*(3), 208-217. doi:10.1159/000365750

[214] Yang, M. H., Lee, K. T., et al. (2012). Guggulsterone enhances antitumor

activity of gemcitabine in gallbladder cancer cells through suppression of NF-kappaB. *J Cancer Res Clin Oncol, 138*(10), 1743-1751. doi:10.1007/s00432-012-1254-7

[215] Yip, K. W., & Reed, J. C. (2008). Bcl-2 family proteins and cancer. *Oncogene, 27*(50), 6398-6406. doi:10.1038/onc.2008.307

[216] Yue, Q., Gao, G., et al. (2017). Natural Products as Adjunctive Treatment for Pancreatic Cancer: Recent Trends and Advancements. *Biomed Res Int, 2017*, 8412508. doi:10.1155/2017/8412508

[217] Zaini, R., Clench, M. R., et al. (2011). Bioactive chemicals from carrot (Daucus carota) juice extracts for the treatment of leukemia. *J Med Food, 14*(11), 1303-1312. doi:10.1089/jmf.2010.0284

[218] Zhang, J., Du, J., et al. (2016). Down-regulation of STAT3 expression using vector-based RNA interference promotes apoptosis in Hepatocarcinoma cells. *Artif Cells Nanomed Biotechnol, 44*(5), 1201-1205. doi:10.3109/21691401.2015.1029628

[219] Zhang, J., Li, L., et al. (2015). Effects of Hypoxia, Surrounding Fibroblasts, and p16 Expression on Breast Cancer Cell Migration and Invasion. *J Cancer, 6*(5), 430-437. doi:10.7150/jca.11353

[220] Zhong, F., Yang, J., et al. (2015). Guggulsterone inhibits human cholangiocarcinoma Sk-ChA-1 and Mz-ChA-1 cell growth by inducing caspase-dependent apoptosis and downregulation of survivin and Bcl-2 expression. *Oncol Lett, 10*(3), 1416-1422. doi:10.3892/ol.2015.3391

[221] Zinczuk, J., Zareba, K., et al. (2018). p16, p21, and p53 proteins play an important role in development of pancreatic intraepithelial neoplastic. *Ir J Med Sci, 187*(3), 629-637. doi:10.1007/s11845-018-1751-z