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ANTICANCER ACTIVITY OF GUGGULSTERONE IN MULTIPLE MYELOMA CELLS

BY

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ABSTRACT

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Title: Anticancer Activity of Guggulsterone on Multiple Myeloma cells

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Guggulsterone (GS), a phytosteroid derived from guggul plants' gum resin, has anti-inflammatory and antioxidant properties. Although, the GS is found to have cytotoxic effects in Multiple Myeloma (MM) cells, the mechanisms of activity have not been described yet. Also, the GS-dependent expression of the High Mobility Group Box-1 (HMGB-1) protein, an attractive target for cancer therapy and its involvement in the JAK/STAT signaling pathway in MM is yet to be discovered. In this study, we aimed to investigate the anticancer activity of GS in various MM cell lines. GS treatment of MM cells resulted in inhibition of cell viability via loss of mitochondrial membrane potential, activation of caspases, and cleavage of Poly (ADP-ribose) polymerase (PARP). Moreover, GS downregulates the expression of HMGB-1 via JAK/STAT signaling pathway and suppresses the constitutive activation of the STAT3 signaling pathway. This study highlights the anticancer potential of GS in MM and the underlying molecular mechanisms responsible for its effect on the pathogenesis of this malignancy.

DEDICATION

To the two who gave me life, and the one who gave me wings

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1. Multiple Myeloma (MM)
2. Guggulsterone (GS)
3. High Mobility Group Box-1 (HMGB-1)
4. Poly (ADP-ribose) Polymerase (PARP)
5. Monoclonal Gammopathy of Undetermined Significance (MGUS)
6. Interleukins (IL)
7. Insulin-like Growth Factor-1 (IGF-1)
8. Transforming Growth Factor (TGF)
9. Toll-Like Receptor 4 (TLR4)
10. Receptor for the Advanced Glycation End Product (RAGE)
11. Signal Transducer Activator of Transcription (STAT)
12. Janus Kinases (JAK)
13. Adenosine triphosphate (ATP)
14. Mitochondrial Outer Membrane Permeabilization (MOMP)
15. X-linked mammalian Inhibitors of Apoptosis Protein (XIAP) (XIAP)
16. Light Chain-3 (LC3-II)
17. Farnesoid X receptor (FXR)
18. Cyclin-dependent Kinase Inhibitor (CKI)
19. Apoptotic Protease Activating Factor 1 (Apaf-1)

1. INTRODUCTION

Cancer, abnormal growth of cells, is high morbidity and high mortality disease. Millions of people are diagnosed with cancer every year, and it is one of the major leading cause of death worldwide. By 2030, the number of cancer cases is estimated to be 21 million globally (Ijaz et al., 2018). In the United States, 1,898,160 new cancer cases and 608,570 cancer deaths are expected to be reported in 2021 (Siegel et al., 2021). External factors such as smoking, radiation, pollutants in edibles, air, and other infectious agents, and internal factors such as immune system, hormonal disorders, and genetic mutations are some known causes of cancer. Lung cancer is the leading cause of cancer deaths in men, whereas breast cancer is the leading cause of cancer deaths among women. This applies to Qatar and world statistics (Global Cancer Observatory, 2021). Other common types of cancer are prostate, kidney, leukemia, liver, thyroid, and skin cancer (Song et al., 2015).

Hematological cancers are initiated in the bone marrow, which is characterized by uncontrolled growth of abnormal blood cells that overtake normal cells' growth and alters these cells' functions. The three major categories of hematological malignancies are leukemia, lymphoma, and myeloma. Of the three, leukemia is the most aggressive and fast-growing cancer that is often diagnosed among adults over 55 years and children under 15 years old. On the other hand, lymphoma occurs when lymphocytes multiply abnormally and start to aggregate in tissues and lymph nodes. Hodgkin lymphoma and non-Hodgkin lymphoma are among common lymphoma observed in humans. Furthermore, cancer in plasma cells is known as myeloma, where antibody production is compromised, and immunity function is weakened (Firth et al., 2019); (Lewis et al., 2020) (Davis et al., 2014).

In Qatar, according to 2020 statistics, total cases of leukemia accounts for 6.3%, non-Hodgkin lymphoma for 5.9%, and MM for 1.3% for both genders of all ages (Globocan, 2020). Increased cancer mortality rates have fueled the pursuit of new treatments and anticancer agents. The use of complementary and alternative medicine has been one of the most promising approaches to reduce the symptoms and minimize conventional cancer treatments' side effects. Guggulsterone (GS), an oleogum resin obtained from guggul trees found in Pakistan, India, and Bangladesh, has been known to treat various conditions such as rheumatism, atherosclerosis, hypercholesterolemia, and obesity, among others. This study aims to describe the anticancer activities of GS and the molecular mechanisms mediating its effects in MM cells.

2. LITERATURE REVIEW

2.1. Multiple Myeloma

MM is a cancer that forms in a type of white blood cells in the bone marrow called plasma cells. Overgrowth of plasma cells crowds the cell population in the bone marrow leading to low blood count levels. As MM progresses, patients develop anemia, hypercalcemia, extensive skeletal destruction, abnormal bleeding, and renal failure. Also, a low level of platelets (thrombocytopenia) is often reported in MM patients and is the cause of increased bruising, bleeding, and inability to fight infections (Firth & contributors, 2019).

MM is the third most common hematological malignancy after non-Hodgkin lymphoma and is associated with significant morbidity due to its end-organ destruction. This disease is more likely to affect the older population and has a higher incidence rate in the African American population. In the Western world, the diagnosis of MM is usually observed more in men of age 66-70 years, while around 37% of patients being younger than 65 years (Kazandjian, 2016). Also, exposure to radiation, asbestos, pesticides, benzene, and other chemicals used in various industries have been identified as the major risk factors in the development of MM (Sergentanis et al., 2015).

MM killed approximately 100,000 individuals globally, with an age-standardized death rate of 1.5 per 100,000 persons. Between 1990-2016, MM cases' incidence increased by 126% globally, and deaths reached to 94% (Cowan et al., 2018). In Qatar, MM has a 1.3% incidence rate and 2.1% mortality rate, of which most of the cases are notable among older men ("Globocan," 2020).

The pathogenesis of MM is complex and involves intricate interaction between MM cells and the microenvironment of the bone marrow. It begins as monoclonal gammopathy of undetermined significance (MGUS) and progresses towards

asymptomatic myeloma. Finally, it transforms to symptomatic myeloma, resulting in bone marrow infiltration and osteolytic lesions. The bone marrow microenvironment in MM is marked by the secretion of various inflammatory mediators such as Interleukins (IL), Insulin-like Growth Factor-1 (IGF-1), and Transforming Growth Factor (TGF) that sustain the adhesion, proliferation, and migration of MM cells through several intracellular signaling pathways (Musolino et al., 2017; Peng et al., 2020). The production of cytokines such as IL-6 induces the survival of MM cells and provides protection against drug-induced apoptosis via JAK/STAT or/and PI3K/AKT signaling pathway (Johnson et al., 2012). It is reported that levels of cytokines are associated with the progression and severity of the diseases and can take part in drug resistance (Dehghanifard et al., 2018). Furthermore, in recent years, an emerging role of the High Mobility Group Box-1 (HMGB-1) protein in promoting cancer cell proliferation metastasis and invasion has been recognized (Cheng et al., 2020). In MM, high levels of HMGB-1 are detected and are linked with a poor prognosis of MM. Therefore, studying the function and mechanism of HMGB-1 in MM is crucial to understand its pleiotropic role in the pathophysiology of other hematological malignancies and solid tumors (Guo et al., 2018)

2.2. High-Mobility Group Box 1

The name "HMG" is derived from the high electrophoretic mobility on polyacrylamide gels. It is a member of the HMG family, which also includes HMGB-2 and -3 proteins. HMGB-1 is the most abundant and conservative member of the HMG family and is primarily a nuclear non-histone chromosomal binding protein (Martinotti et al., 2015). It binds to the DNA and is involved in chromosome remodeling, gene transcription, and DNA damage repair (Guo et al., 2018).

The HMGB-1 molecule is a single polypeptide chain with 215 amino acids. The

protein comprises three main structural domains, i.e., N terminal A-box (AA 1-79), central B-box (AA 89-162), and acidic C tail (AA 186-215). The A and B boxes are involved in protein binding to DNA and promote the DNA chain's twisting and folding. The A-box is also involved in the induction of anti-inflammatory effects, while the B-box constitutes binding sites for Toll-Like Receptor 4 (TLR4) and Receptor for the Advanced Glycation End Product (RAGE). Hence, both domains play an essential role in HMGB-1 proinflammatory effects. (Martinotti et al., 2015); (Wang et al., 2016). The C-terminal region is a key player in transcription stimulation as it is enriched with negatively charged aspartic and glutamic acid. Furthermore, within HMGB-1, three cysteines (Cys) residues (two in A and one in B) contribute to the biological activities of the extracellular HMGB-1 (Yang et al., 2015).

HMGB-1 has multiple functions in the immune system such as tissue healing, regeneration and has a protective role in cancer immunity in a tumor microenvironment (Son et al., 2020). It also induces cell proliferation, increases expression of cell-surface molecules associated with inflammation, and cytokine production. Several pieces of evidence highlight the role of HMGB-1 in diseases such as sepsis, rheumatoid arthritis, stroke, and many more. In addition, HMGB-1 activates and promotes the maturation of dendric cells, thereby sustaining long-term repair programs and acting in autocrine/paracrine manner. Studies have reported the HMGB-1 induced arthritis in mice and is upregulated in active brain lesions in multiple sclerosis. Therefore, HMGB-1 is actively involved in the pathogenesis of various diseases via pleiotropic effects, ranging from angiogenesis, inflammation, and DNA damage repair to cancer development, invasion, metastasis, and drug resistance. (Guo et al., 2013); (Martinotti et al., 2015).

HMGB-1 exists as a nuclear and cytoplasmic protein as well as a secreted

protein mainly by monocytes and macrophages. Also, HMGB-1 can be leaked out by necrotic cells where it triggers inflammation. The nuclear HMGB-1 binds to DNA and promotes the assembling of protein on specific DNA sites. On the contrary, when it is secreted outside the cell, it can bind to high-affinity receptors such as RAGE and mediates inflammation (Tang et al., 2010). It translocate from the nucleus to the cytosol under stress conditions such as hypoxia, inflammation, and external agents such as cytokines, chemokines, and other factors. In cancers, epigenetic modifications occur and play an essential role in its progression. Several post-translational modifications such as acetylation, oxidation, methylation, and phosphorylation are responsible for the modulation of HMGB-1 structure, its subsequent biological functions, and localization (Zhou et al., 2014)

An increasing body of evidence points out the association of HMGB-1 with various hallmarks of cancer such as angiogenesis, apoptosis, tissue invasion and metastasis, enhanced inflammation, and insensitivity to growth inhibitors (Martinotti et al., 2015); (Venereau et al., 2016)). This attributes an emergent oncogenic role for HMGB-1 gene as it is shown to overexpress in serum levels of solid tumors, prostate, breasts, lung, ovarian, and pancreatic cancers (Martinotti et al., 2015); (Zhou et al., 2014); (Xu et al., 2019). Collectively, these studies make HMGB-1 a critical molecular target in cancer development and progression. Nevertheless, the role and regulation of HMGB-1 expression in MM require more investigations.

2.3. JAK/STAT Signaling Pathway

Oncogenic transcription factors can serve as connecting points in multiple signaling pathways and can be major players in gene expression that drive MM cells' malignancy (Lambert et al., 2018). One such family of transcription factors is the Signal Transducer Activator of Transcription (STAT), a predominant intracellular signaling

protein involved in the regulation of tissue invasion, metastasis, cell survival, proliferation, and angiogenesis. In MM, STAT3, like other STAT family members, is present in the cytoplasm and relays signals from growth factor cytokines and receptors. Structurally, it includes an N-terminal coiled-coiled domain, a DNA binding domain, an Src homology 2 (SH2) domain, and a C-terminal transactivation domain. For the activation of the STAT3 signaling pathway, cytokines and growth factors prompt the tyrosine kinases activity of Src or Janus kinases (JAK) receptors, resulting in tyrosine phosphorylation of intracytoplasmic receptor chains. Through this process, STAT3 monomers form dimers and translocate to the nucleus. In the nucleus, activated STAT3 dimers bind to DNA and initiate gene transcription (Harrison, 2012); (Liu et al., 2014).

The JAK/STAT pathway is a pleiotropic cascade that plays a significant role in various physiological processes, including immune function, hematopoiesis, cell growth, and apoptosis (Hammarén et al., 2019). Accumulating evidence indicates that alterations in JAK/STAT signaling can result in disease and cancer, putting members of the JAK/STAT pathways as potential targets for therapeutic development (Hammarén et al., 2019); (Akhtar et al., 2019). The critical parts in this pathway are JAK, STAT, and cell surface receptors. JAK has been identified as a unique class of tyrosine kinases and contains a catalytic and kinase-like domain. They are cytoplasmic and receptor-associated proteins. For instance, when a cytokine binds to its receptor, the receptor is dimerized, and its associated proteins are activated. This, in turn, activates the tyrosine kinase activity of JAKs, whereby activated JAKs phosphorylate each other along with the tyrosine residues. Further, this establishes the docking of STAT3 via the SH2 domain, which activates and phosphorylates STAT3 (Harrison, 2012). This suggests that inactive STAT3 is recruited to the activated receptor and is phosphorylated at the Tyr705 site by JAK or Src. In addition to the Tyr705

phosphorylation. STAT3 can also be activated via phosphorylation of serine (Ser 727) which is commonly regulated by protein kinase C, CDK5, or mitogen-activated protein kinases (MAPK). Another mechanism of STAT3 activation is by histone acetyltransferase on a single lysine residue (Lys685). The acetylated STAT3 enhances the ability of STAT3 dimers in DNA binding and its transcriptional activity (Harrison, 2012).

Various hematological malignancies and solid cancers such as breast, colon, and cervical cancers have reported the activation of STAT3 (Brooks & Putoczki, 2020). Also, hematological malignancies, specifically MM, exhibit the activation of the JAK/STAT signaling pathway. This activation is triggered by the phosphorylation of STAT induced by IL-6 signaling in the bone marrow (Chong et al., 2019). Indeed, it has been established that MM cells depend on cytokines, especially, IL-6 for their continuous growth. In addition, MM patients often exhibit high levels of activated STAT3 while bone marrows from healthy donors do not demonstrate STAT3 activation (Catlett-Falcone et al., 1999). This is because the bone marrow stromal cells produce IL-6, which may be secreted via MM cells, promoting the activation of STAT3. In some cases, STAT3 can also be activated by cell-cell interactions. Also, in large myeloproliferative diseases such as MM, JAK2 is activated through point mutation in IL-6 autocrine and paracrine loops and acts as a driving force for the activation of STAT3 (O'Shea et al., 2015). This highlights the importance of Jak inhibitors in targeted therapy, where pharmacological inhibition of JAK can lead to the decrease in the phosphorylation of STAT3, which eventually decreases the proliferation and survival of MM cells (Oshiro et al., 2001).

An important question is whether STAT3 activation in MM cells is a direct contributor to cancer pathogenesis or is merely an activated bystander transcriptional

factor with no physiological consequence. Using immunohistochemical staining (IHC), high levels of tyrosine phosphorylation of STAT3 (a marker for STAT3 activation) were detected in MM patients (Chong et al., 2019). Also, preclinical data suggested high drug resistance when STAT3 is aberrantly activated. Furthermore, considerable evidence suggests that activated STAT3 promotes angiogenesis, suppresses the host's surveillance of tumors, and supports dysregulation of growth and survival (Turkson, 2004). Indeed, it has been shown that the inhibition of aberrant STAT3 activity can induce growth arrest, and apoptosis of tumor cells in vitro and in vivo (Siddiquee et al., 2007); (Yue & Turkson, 2009). The use of STAT3 inhibitors has depicted anticancer activity in tumor mouse models. This includes promoting the reduction of cancer-inducing immune response and increasing antitumor immunity (Kortylewski et al., 2005); (Yu et al., 2009).

Some critical downstream targets for the oncogenic effect of STAT3 are cyclin D, Bcl-xl, Myc, Mcl-1, and VEGF. In a study by Quintanilla Martinez and others, (Quintanilla-Martinez et al., 2003), the activation of STAT3 and its effect on downstream targets, including cyclin D1, Bcl-xl, Mcl-1, and Bcl-2, was investigated. Findings of the study concluded that downstream targets of STAT3 also play an essential role in the pathogenesis of MM.

In addition, it has been shown that the JAK/STAT signaling pathway can modulate the expression of HMGB-1 in various conditions**.** JAK/STAT activation induces the translocation of HMGB-1 from the nucleus to the cytoplasm and its subsequent release in the extracellular space. On the other hand, the pharmacological inhibition of JAK/STAT prevented HMGB-1 translocation. This underlines the critical role of the JAK/STAT signaling pathway in prompting the cytoplasmic translocation of HMGB-1 and its subsequent secretion in extracellular space, therefore, highlighting

the JAK/STAT pathway as a potential molecular target for inhibiting HMGB-1 release (Lu et al., 2014). However, the role of HMGB-1, its association with the JAK/STAT signaling pathway, and IL-6 in MM has not been fully elucidated.

2.4. Apoptosis, Necrosis and Autophagy

2.4.1. Apoptosis

Apoptosis or programmed cell death is an ordered and tightly regulated cellular process in physiological and pathological conditions. It plays essential roles in several biological events such as homeostatic maintenance of tissues, morphogenesis, and removal of unwanted cells. Dysregulated apoptosis is associated with diseases such as cancer, neurodegenerative and autoimmune disorders. Several conditions and stimuli are responsible for triggering apoptosis. For instance, chemotherapeutic drugs and irradiation cause DNA damage and lead to apoptotic cell death while other cells may remain unaffected. Apoptosis may be initiated by one of the two separate pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Figure 1). Both pathways converge on the same execution phase, which is initiated by the cleavage of a family of conserved Cysteinyl Aspartate Specific Proteases (Caspases), specifically, caspase 3 (Ashkenazi, 2008); (Pfeffer & Singh, 2018).

Extrinsic apoptosis signaling pathways involve transmembrane receptor-mediated interactions. Some of the characterized ligands and corresponding death receptors are FasL/FasR, TNF-a/TNFR1, Apo2L/DR4, and others. Upon binding to the ligand, cytoplasmic adapter proteins of the corresponding death receptor are recruited, which bind with the receptors. For instance, Fas ligand binds to Fas receptor, which results in the binding of the FADD adapter protein. Through dimerization of the death receptor domain, FADD then links with procaspase 8, and a death-inducing signal complex (DISC) is formed. This formation further results in the autocatalytic activation

of procaspase 8. The executive pathway of apoptosis is triggered as a result of activation of caspase 8 (Krautwald et al., 2010); (Elmore, 2007); (Matthews et al., 2012).

In contrast, the intrinsic apoptotic pathway is initiated by a range of endogenous and exogenous stimuli that involve mitochondrial events. These stimuli produce intracellular signals that may act positively or negatively. In negative signals, growth factors, hormones, and cytokines are absent, which leads to the failure of cell survival and initiation of apoptosis. On the contrary, stimuli from positive signals are viral infections, hypoxia, radiation, toxins, and free radicals (Elmore, 2007). Changes that occur in the mitochondrial membrane due to these stimuli result in the opening of mitochondrial permeability transition (MPT) pores and loss of membrane potential. Bcl-2 family bind to the mitochondrial membrane. Bcl-2 family governing the MMP can be either proapoptotic (Bax, Bak, Bid) or antiapoptotic (Bcl-2, Bcl-x, Bcl-xl). Bcl-2 proteins also regulate the release of cytochrome-c from the mitochondria by altering the Mitochondrial Membrane Potential (MMP). Cytochrome-c then binds to apoptotic protease activating factor 1 (Apaf-1) as well as procaspase 9, thereby forming an "apoptosome" or a complex. The apoptosome hydrolyzes adenosine triphosphate (ATP), cleaves, and activates caspase 9. Caspase 9, an initiator caspase, then cleaves and activates executioner caspases 3,6 and 7, resulting in apoptosis (Loreto et al., 2014). These execution caspases activate cytoplasmic endonuclease, which results in the degradation of the nuclear material, and cytoplasmic proteases that degrade cytoskeletal and nuclear proteins. Executioner caspases are also responsible for cleaving various other substrates such as PARP, which ultimately results in biochemical and morphological changes of the apoptotic cells. Cancer cells become resistant to apoptosis through the expression of antiapoptotic proteins such as Bcl-2 and the downregulation of proapoptotic proteins such as Bax. Bid, another member from the

Bcl-2 family, is reported to be the bridge between extrinsic and intrinsic signaling pathway as reported in several studies (Segal et al., 2007); (Roy & Nicholson, 2000). Intact Bid (uncleaved) is not able to induce apoptosis. However, in the extrinsic pathway of apoptosis, Bid is cleaved by caspase 8, resulting in the generation of a protein termed truncated Bid (tBid), which can induce mitochondrial outer membrane permeabilization (MOMP). Therefore, Bid's cleavage into its truncated form establishes the link between extrinsic and intrinsic apoptosis pathways (Kantari & Walczak, 2011).

The inhibition of apoptosis proteins (IAP) is important regulator of apoptosis due to their presence in extrinsic and intrinsic pathways. The X-linked mammalian inhibitors of apoptosis protein (XIAP) and survivin are two main known members of IAP. These proteins are widely regarded as therapeutic targets for various diseases, including cancer (Elmore, 2007); (Reed, 2000).

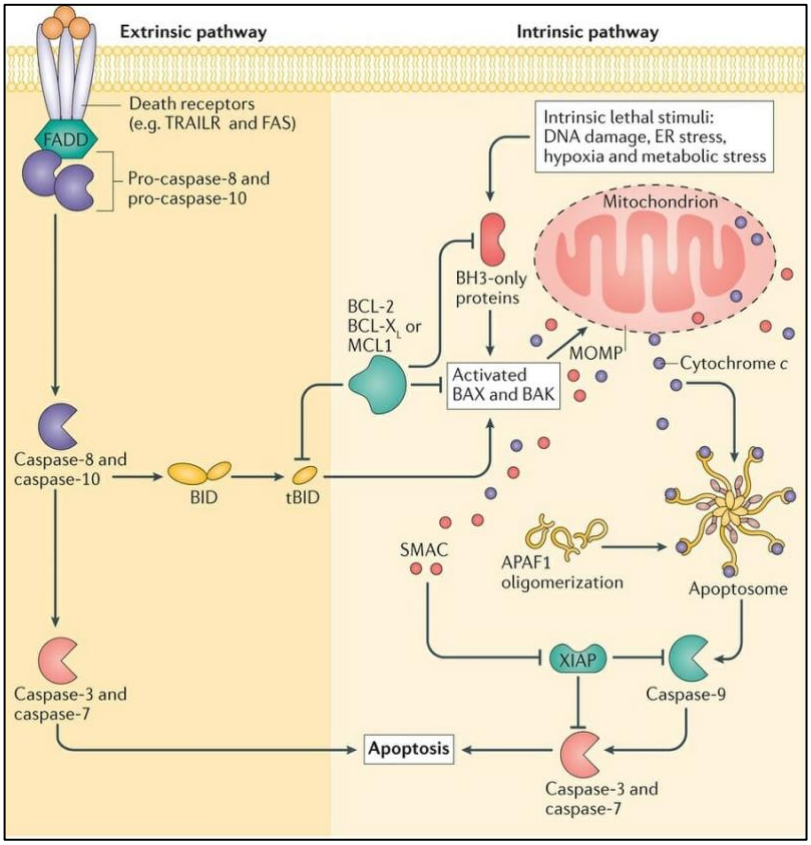

Figure 1: Extrinsic and intrinsic pathways of apoptosis. Source: (Editors, 2017)

2.4.2. Necrosis

Cell death resulting from membrane homeostasis disruption and the loss of ion and water balance due to deregulation of sodium/potassium pumps is termed necrosis (Ferrer & Vidal, 2017). It occurs through an unorderly, passive cellular explosion in response to an overwhelming trauma such as ischemia, hypoxia, hypoglycemia, extreme changes in the temperature, toxin exposure, and nutrients deprivation. These necrotic cells are often characterized by organelles' swelling (such as the mitochondria and the endoplasmic reticulum), plasma membrane rupture, and cell lysis. Inflammatory responses often follow necrotic cell death due to the release of cell contents such as nucleic acid and proteins. This cell death mode does not involve the activation of caspases and is mediated in response to cell damage (Syntichaki & Tavernarakis, 2002). Moreover, PARP overexpression can deplete adenosine triphosphate (ATP) and lead to subsequent necrosis. However, like apoptosis, necrosis may also occur in a programmed manner, a mechanism called necroptosis.

Cells can undergo necroptosis when apoptosis is blocked, and TNF becomes involved in triggering cell death. Until recently, several studies suggested the role of necroptosis in promoting tumor growth. However, it was reported that necroptosis might play roles in both tumor suppression and promotion (Najafov et al., 2017). A critical factor for tumor growth is angiogenesis; however, in solid tumors of certain sizes, vascularization is minimal in the core regions and results in tumor necrosis. This is because tumor cells tend to experience metabolic stresses such as nutrient deprivation and hypoxia in inadequate vascularization areas. Due to the inflammatory responses produced in necrotic cell death, the antitumor immunity in the tumor microenvironment is increased. More specifically, necroptosis due to irradiation or chemotherapy elevates antitumor immunity. For instance, radiation-induced necrosis improves prognosis and

elevates antitumor immunity by releasing the proinflammatory mediator HMGB-1. This suggests that necroptosis may mediate tumor suppression (Liu & Jiao, 2019).

2.4.3. Autophagy

Autophagy is a process of regulated mechanism of degradation and recycling cellular components. There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Chaperone-mediated autophagy involves the direct recognition of target proteins by the cytosolic chaperones and their immediate transport to the lysosome membrane, whereas, in microautophagy, the lysosome membrane engulfs minute portions of the cytoplasm. Finally, macroautophagy, also termed autophagy, involves the formation of three major structures, including the phagophore, autophagosome, and autophagolysosome. The phagophore engulfs cytosolic materials and forms the closed autophagosome. One of the widely used autophagosome markers is the light chain-3 LC3-II protein. The fusion of the autophagosome with lysosomes leads to the formation of auto phagolysosomes responsible for degrading engulfed materials, including LC3-II. Autophagy related gene (Atg) family controls the various autophagic stages such as formation, regulation, elongation, and multimerization of phagophores, cargo selection, and lysosome fusion (Wesselborg & Stork, 2015)

Impaired or excessive autophagy can result in cell death, indicating its role as a double-edged sword in cancer. When cancer is initiated, autophagic processes can suppress the growth and progression of tumors. However, deregulation of autophagy results in genomic instability and necrosis, thereby inducing inflammation that favors tumor initiation. Usually, Atg is absent in tumors, and upon mutation, specific key proteins of autophagy such as Beclin-1 can be correlated with the development of various cancers. MM cells are specifically more reliant on autophagy for the

degradation and recycling of excess protein aggregates. Thus, MM cells are reported to display an unusual activity of basal autophagy. In a study by Jung and others (Jung et al., 2015), it was reported that MM cells have high immunoreactivity against autophagic biomarkers such as beclin-1 and p62, which promote MM cell proliferation. Additionally, autophagy in MM exhibits a protective effect when subjected to drug treatment. Inhibition of this protective autophagy via different targets can enhance the sensitivity of MM cells to drug treatment (Yun et al., 2017).

In recent years, bortezomib and doxorubicin have become irreplaceable drugs for MM, and resistance to these drugs is a major drawback in MM therapy. Constant renewable therapy techniques and novel drug treatments are required to overcome drug resistance in MM. Ongoing conventional treatments cannot suffice the uprise in the numbers of drug-resistant cases in MM, an uproar on the use of complementary and alternative medicine for MM therapy, among other cancer therapies.

2.5. Conventional Medicine

Conventional treatment for MM includes chemotherapy, targeted therapy, biological therapy, bone marrow transplant, and radiation therapy. Chemotherapy uses drugs to treat cancer by impeding the ability of MM cells to grow and spread. For instance, Bortezomib (BTZ), known by its brand name Velcade, has been regularly used as a chemotherapeutic agent, either alone or in combination with other agents, to treat MM (Landowski et al., 2005); (Field-Smith et al., 2006). Also, high dose chemotherapy (HDT) is administrated to MM patients, which has led to an overall extended survival rate in younger patients compared to conventional chemotherapy. However, with the continuous use of drugs such as BTZ, Thalidomide, or Lenalidomide, MM cells develop drug resistance, challenging MM therapy. For relapsed and refractory patients, the use of proteasome inhibitors (PIs) and

immunomodulatory drugs (IMIDs) are used to improve patients' overall survival.

Furthermore, targeted therapy is also used as a conventional form of treatment for MM, which uses drugs to target specific genes and proteins involved in cancer cells' growth and survival. Biological therapy enhances the body's immune system to identify and kill cancer cells. In bone marrow transplant procedure, the diseased bone marrow is replaced with a blood stem cell transplant that forms a new and healthy marrow. Lastly, radiation therapy uses beams of energy, such as protons and X-rays, to inhibit MM cells' growth. Despite the significant advancements made in chemotherapeutic agents and cell therapy, major limitations such as toxicity, relapsing, and drug resistance still exist (Bladé et al., 2010); (Tosi, 2013); (Loke et al., 2020).

Over time, the way of selecting primary treatment for MM patients has changed significantly. The type of treatment chosen is based on age, comorbidities, and individual patient performance. Frailty and older age are more susceptible to life-threatening side effects of conventional medicine for MM patients (Palumbo et al., 2011). Older patients are thought to benefit from autologous stem cell transplantation and have a survival rate of 2-3 years. As mentioned previously, most MM patients are observed to be above the age of 55, which makes them most vulnerable to the disease and the adverse side effects of the conventional MM treatment method. This calls for complementary and alternative medicine that is currently a hot topic among scientists, researchers, clinicians, and cancer patients. MM patients are more inclined to use complementary and alternative medicine alone or in combination with conventional medicine (Palumbo et al., 2015); (Frass et al., 2012).

2.6. Complementary and Alternative Medicine

Complementary and alternative medicine (CAM) plays an integral role in serving almost 80% of the world's population, and the use of such formulations can be

dated back to millennia-old medical practices such as folk medicine. CAM encompasses a broad range of therapeutic approaches, including herbal medicines, homeopathic remedies, and essential oil and dietary supplements. Polyphenols from green tea, grape seed/skin, anthocyanin, and pigments from many flowers, algae, fruits, and vegetables were shown to be safe and effective in cancer prevention and therapy. A common property of many of these compounds is their antioxidant/free radical scavenging ability. However, some are also involved in preferentially inducing high free radical formation to cause the killing/elimination of cancer cells. Hence, the immense number of resources contained in diverse plant species is often underestimated (Adams & Jewell, 2007).

There is a strong correlation between the development of cancer and dietary habits. Various bioactive compounds in food can counteract cancer and improve health. Identification of phytochemicals is now being used for cancer prevention and therapy. The use of phytochemicals is beneficial as they affect the differentiation, proliferation, angiogenesis, and apoptosis evasion of cancer cells. They reduce the harmful effects of chemotherapy and radiation therapy (Bose et al., 2020). Several studies have highlighted the role of phytochemicals in interfering with JAK/STAT pathway in malignant cells. (Siveen et al., 2014); (Singh et al., 2014).

2.7. Commiphora mukul

Commiphora mukul is a small thorny plant indigenous to India, Pakistan, and parts of the Near East (Mesrob et al., 1998). The flowers are red, and the fruit is oval in shape and pulpy in nature (Figure 2). It is also known as Guggul gum, Guggal, Guggulsterone, Guggulu, and gum Guggul. The guggul gum resin has been used in Ayurvedic medicine for several years to treat bone fractures, inflammation, abdominal disorders, rheumatism, and obesity.

Figure 2: Commiphora mukul. Source: ("Guggul Resin Powder," 2020)

Guggulsterone (GS), (4,17(20)-pregnadiene-3,16-dione), is a phytosteroid found in the gum resin of the guggul plant, *Commiphora mukul*. It can exist as two stereoisomers, E-Guggulsterone, and Z-Guggulsterone. GS is known to possess anti-inflammatory, antioxidant, hypocholesterolemia, and hypoglycemia activities, making it an important agent in preventing several disorders. This polyphenol has been traditionally used to treat obesity, diabetes, osteoarthritis, inflammatory bowel disease, hyperlipidemia, and solid tumors (Yamada & Sugimoto, 2016). One of the common mechanisms that GS acts through is its proapoptotic effect, thereby inducing apoptosis in various cancers while showing minimal to no effect on normal cell viability. It is found that Bax and Bak mediate GS-induced caspase-dependent apoptosis. When treating cancer cells with GS, the expression of proapoptotic proteins is increased while the expression of antiapoptotic proteins is decreased (Singh et al., 2005). Furthermore, Farnesoid X receptor (FXR) acts through cell migration and invasion. It has been reported that GS blocks the action of FXR and inhibits the metastasis of cancer cells. In addition, studies have reported that GS exhibits anti-angiogenic and antitumor effects via suppression of nuclear factor-kB (NF-kB) and STAT-3 activity (Ahn et al., 2008).

The anticancer activity of GS on hematological malignancies, such as MM, is poorly investigated. However, a study conducted by (Samudio et al., 2005) found that

GS induces apoptosis in leukemic cells via a caspase-independent mechanism that involves an increase in ROS generation and a decrease in the phosphorylation of extracellular signal-regulated kinase (ERK). Another study conducted by (Bhat et al., 2017) showed that GS inhibited the proliferation of a variety of cancer cells by decreasing the levels of cyclin D1 and cell division control protein 2 (CDC2) while increasing the levels of cyclin-dependent kinase inhibitor (CKI) p21 and CKI p27. The inhibition of cell proliferation was associated with the induction of apoptosis by activation of caspase-cascade, Poly ADP-ribose polymerase (PARP) cleavage, and downregulation of antiapoptotic effectors. Taking together, these observations indicate that GS may potentially have a cancer chemo preventive and therapeutic activity by targeting survival pathways such as PI3-kinase/AKT, NF-κB, and JAK/STAT that are involved in the control of cell growth and inflammatory responses via regulation of antiapoptotic and inflammatory genes (Bhat et al., 2017).

Therefore, the current study aims at elucidating the molecular mechanism underlying the antiproliferative effects of GS in various MM cells, and most importantly, to determine the effect of GS on regulating the JAK/STAT signaling pathway and the expression of HMGB1.

3. RATIONALE

MM is a devastating and incurable disease that affects the bone marrow (BM) in different areas of the body, such as the spine, skull, pelvis, and ribs. The early diagnosis of MM is often challenging as it does not show symptoms until it reaches advanced stages or after a routine blood test. Clinical features of MM include bone pain with osteolytic lesions, shortness of breath, weakness, and fatigue, anemia, recurring infections, kidney failure, and less commonly bleeding gums, bruising, and nosebleeds. The etiology of MM is unknown but is thought to arise from an asymptomatic pre-malignant stage of clonal plasma cell proliferation termed monoclonal gammopathy of undetermined significance (MGUS) and is associated with excessive production of immunoglobulins (M-proteins) in the blood. Approximately 1 in every 100 people with MGUS develops MM. Patients with MM have a decreased quality of life caused by physical incapacity and the feeling of living with an incurable illness for a long time. This study aims to investigate the effect of a phytosteroid, Guggulsterone, on MM cells as an effort to introduce novel targets to combat MM. The outcomes of this project would provide a better understanding of MM and shall have significant clinical implications that could result in the development of innovative strategies for targeted therapeutic intervention for the treatment of MM.

4. HYPOTHESIS

In this study we hypothesize:

1. GS treatment mediates the downregulation of HMGB-1 in MM cells
2. GS suppresses the activation of JAK/STAT pathway, which downregulates the expression of HMGB-1 in MM cells.
3. GS inhibits IL-6 induced activation of STAT3 and downregulates HMGB-1 in MM cells.

5. OBJECTIVES

The specific aims of this study are:

1. To assess the effects of GS in various MM cell types
2. To determine whether GS-induced apoptosis occurs via intrinsic or extrinsic pathways of apoptosis
3. To investigate the impact of GS on JAK/STAT signaling pathway and expression of HMGB-1
4. To elucidate the effect of GS on constitutive and IL-6 mediated activation of STAT3.

6. MATERIALS AND METHODS

6.1. Reagents and antibodies

Z-Gugglesterone (GS) (TOCRIS) was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. JAK inhibitor AG-490 was dissolved in DMSO and used at a concentration of 25 μM. Cell Counting Kit-8 (CCK 8) and N-acetylcysteine (NAC) were purchased from Signal Chemical Co. (St.Louis, USA). Z-VAD-FMK was purchased from Calbiochem (San Diego, USA). Live/dead cell viability/cytotoxicity kit was purchased from Molecular Probes (USA). Caspase-3/7 green detection reagent was purchased from Invitrogen. Antibodies against pH2AX, cleaved caspase-8, Bcl-2, Bax, cytochrome-c, caspase-3, cleaved caspase-3, PARP, caspase-9, LC3, p62, Atg7, Atg5, HMGB-1, phospho-STAT-3, STAT-3, Bcl-xl, cyclin-D1, SHP-1 were purchased from Cell Signaling Technology (Beverly, USA) and GAPDH, β-actin and HSP60 from Santa Cruz Biotechnology, Inc (Santa Cruz, USA). Other antibodies, including BID, P27, XIAP were purchased from Abcam (Cambridge, UK). FITC Annexin V apoptosis detection kit I, Apo-Direct kit, Fixation/Permeabilization solution kit, BD MitoScreen (JC-1), BV421 mouse anti-γH2AX (pS139), PE rabbit anti-active caspase-3, and Alexa Fluor 700 mouse anti-cleaved PARP (Asp214) antibodies were purchased from BD Biosciences (San Jose, USA). HMGB-1 ELISA kit was purchased from Biomatrik. STAT3 siRNA and negative control siRNA were purchased from Life Technologies and Qaigen, respectively. RPMI 1640, fetal bovine serum (FBS), Penicillin Streptomycin (PenStrep) were purchased from Gibco Thermo Scientific Hyclone (California, USA).

6.2. Cell culture

Human RPMI-8266, U266-B1, MM.1S cells lines were obtained from ATCC, USA and and Peripheral Blood Mononuclear Cells (PBMC) from healthy donors were received

from Dr. Maysaloun Merhi at Hamad Medical Corporation and cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin at 37°C and 5% $C0_2$.

6.3. Cell Viability Assay

10,000 cells were grown in 96 well culture plates and treated with increasing concentration of GS (5, 10, 25, 50 µM). After 24 h, 48 h, 72 h, and 96 h of incubation, 10µl of CCK-8 reagent were added to the wells, and the plate was incubated for 1h at 37ºC. Optical density was measured at 450 nm and calculated as described previously (Prabhu et al., 2018).

6.4. Live/dead assay

U266 cells were seeded at a density of 5 x 10^3 per well in a 6-wells plate and treated with GS for 48 h. After the end of the incubation period, preparation of the live/dead stain was carried out by adding 5µL of ethidium homodimer-1 (EthD-1), and 5µL calcein AM to 10 mL PBS (i.e., final concentrations of 1 µM EthD-1 and 2 µM calcein). The cells were stained with the prepared dye for 1h, visualized, and images were captured using EVOS fluorescence microscope (Life Technologies) at 40X magnification.

6.5. Caspase 3/7 green apoptosis assay

U266 cells were treated with GS for 48 h in a 12 wells plate. After the end of the treatment period, two drops of the caspase 3/7 green detection reagent were added to the cells and incubated for 30 minutes. After the end of the incubation period, the plate was visualized, and images were captured under the EVOS fluorescence microscope.

6.6. Cell Lysis and Protein Immunoblotting

U266 cells after treatment with GS were lysed with laemmli buffer. Quantification of

the proteins was conducted using ND-100 (Nanodrop Technologies, Thermoscientific, USA). After adding of β-mercaptoethanol, 30-60 µg of samples were loaded, proteins were separated by SDS-PAGE and transferred to PVDF membrane. Using various antibodies, these membranes were immunoblotted and developed using chemidoc system (Amersham, Bio-Rad, USA).

6.7. AnnexinV/propidium iodide dual staining

U266 cells were treated with various doses of GS for 48 h. After 48 h, the cells were harvested, washed with PBS, and stained with annexin V-FITC and propidium iodide in 1x annexin binding buffer for 20 minutes. The analysis was done using flow cytometry (BD Sciences) using BD LSRFortessa analyzer, and cells were quantified as live (Annexin $FITC^{-ve}$, PI^{-ve}), early apoptotic (Annexin $FITC^{+ve}$, PI^{-ve}), late apoptotic (Annexin $FITC^{+ve}$, PI^{+ve}), and necrotic (Annexin $FITC^{-ve}$, PI^{+ve}). Early and late apoptosis percentages were added and expressed as apoptosis percentages in the results section (Hussain et al., 2007)

6.8. Measurement of DNA double-strand breaks

U266 cells were treated with increasing concentration of GS for 48, fixed, and permeabilized using BD cytofix/cytoperm plus fixation and permeabilization solution kit. Next, $1x10^5$ cells were stained with 5 µl H2AX (pS139)- Alexa Fluor 647 antibody and double stranded DNA breaks were detected using flow cytometry as described by (Iskandarani et al., 2016).

6.9. Measurement of Membrane Potential (MMP)

To determine the MMP in MM cells treated with GS, JC1 stain kit was used as described previously by (Prabhu et al., 2017). The membrane-permeant JC-1 dye is an indicator of mitochondrial membrane potential and is widely used in apoptosis studies to monitor mitochondrial health. According to the protocol of the manufacturer, cells

were stained briefly with JC-1 stain in the dark. Each sample's MMP was determined using flow cytometry and was denoted by the reduction in red fluorescence. The mean values were plotted in the graph for loss of MMP representation.

6.10. Flow cytometric analysis of active caspase-3 and cleaved PARP

Replicates of U266 cells treated with increasing concentrations of GS were fixed and permeabilized using fixation/permeabilization solution kit. According to the manufacturer's protocol, 0.5 x 10^6 cells in 1x perm-wash buffer were stained with AF700 cleaved PARP and BV605 tagged anti-active caspase-3 (each 5µl) for 30 min and washed twice. Finally, the analysis was carried out via flow cytometry using BD LSR Fortessa analyzer as previously mentioned by (Prabhu et al., 2017)

6.11. ELISA HMGB1

U266 cells were treated with GS for 48 h. After the end of the treatment period, the cells were centrifuged at 1300 rpm for 5 minutes and washed with PBS. The cell supernatant was collected for the experiment. HMGB1 ELISA assay was performed as per manufacturer's protocol. For this assay, Human HMBG1 ELISA kit (Biomatrik) was used.

6.12. Gene silencing using siRNA

4D- Nucleofactor™ System (Lonza) was used for the transfection of U266 cells with STAT 3 siRNA (Life Technologies, California) and control siRNA (Qiagen) according to the protocol. The cells were incubated for 48 h at 37°C. The cells were then lysed and immunoblotted with STAT 3, HMGB1, and HSP60 for equal loading.

6.13. Multiplex Cytokine Analysis

Cytokines measurement was performed using multiplexing array kit was used (BD Bioscience, USA). GS treated U266 cells supernatant was collected, and quantitative

analysis for IL-6 was performed. Antibodies and biotin-conjugated antibodies were added to tubes containing cytokine standards for the concentration curve. Streptavidin-PE was added to the mix after 2 h and subsequent washing with Tween 20 and PBS (PBS/T). After additional 1hr incubation, the samples were washed with PBS/T and required resuspension of beads in PBS containing 10% FBS. This was then analyzed using flow cytometry (BD FACSCalibur, BD Bioscience, USA).

6.14. Statistical Analysis

GraphPad Prism 7 software was used for the statistical analyses. One-way ANOVA evaluated statistical differences between mean values followed by Sidak's posthoc test. Results were represented as mean ± SD, and differences between groups were considered statistically significant when $p \leq 0.05$.

7. RESULTS

7.1. Effect of GS on PBMC and MM cell viability.

The first objective of this study was to determine the effect of GS on MM cells. Thus, U266, MM1S, and RPMI cell lines are treated with an increasing concentration of GS for 24 h, 48 h, 72 h, and 96 h. To assess the cell viability, CCK-8 solution was used. Figure 3 A shows the effect of GS treatment of U266 cells at 24, 48, 72, and 96 hours. Results show a significant decrease in the cell viability in a dose-dependent manner when treated with 0, 5, 10, 25, and 50 µM of GS, respectively. The same pattern was observed in RPMI and MM1S cells (Figure 3B, 3C, respectively). At 24 h, for all cell lines, the half-maximal inhibitory concentration (IC50) was not achieved. However, at 48h, IC50 for U266 cells was observed with 25 and 50 µM GS, whereby the cell viability decreased from 100% to 42.7% ± 0.5 and 29.7% ± 0.9, respectively. In contrast, IC50 for MM1S (59%) and RPMI (48.5%) was observed between 25-50 µM of GS at 72 h. The optimal GS treatment period for this study was fixed at 48 h, and U266 cells were chosen for the remainder experiments in the study as robust effect of GS was observed in U266 cells at 48 h. To determine the effects of GS on healthy cells, PBMC from healthy donors were treated with the same doses of GS mentioned above for 48 h. After the treatment period, CCK-8 solution was added to the cells to assess the cell viability. Results showed that GS does not have any harmful effects on PBMC as there no difference observed in the cell viability when compared to the control and GS treated wells (Figure 3D). CCK-8 works through the utilization of water-soluble tetrazolium salt (WST-8) to quantify the number of live cells by producing an orange formazan dye upon bio-reduction in the presence of an electron carrier. The amount of formazan dye (orange color) generated in the cells by dehydrogenases is directly proportional to the cells' number.

To study the cytotoxicity of GS on U266 cells, live/dead assay was performed. After 48 h of GS treatment, U266 cells were stained with 1 μM EthD-1 and 2 μM calcein AM and incubated for 1 h. Images showed a GS-mediated decrease in U266 viability with maximum death at 50 μM GS (Figure 3E). The live cells stained green because Calcein AM easily enters the cells via diffusion and was converted to calcein by intercellular esterase. Meanwhile, dead or damaged cells take up EthD-1, and they were observed to be stained red.

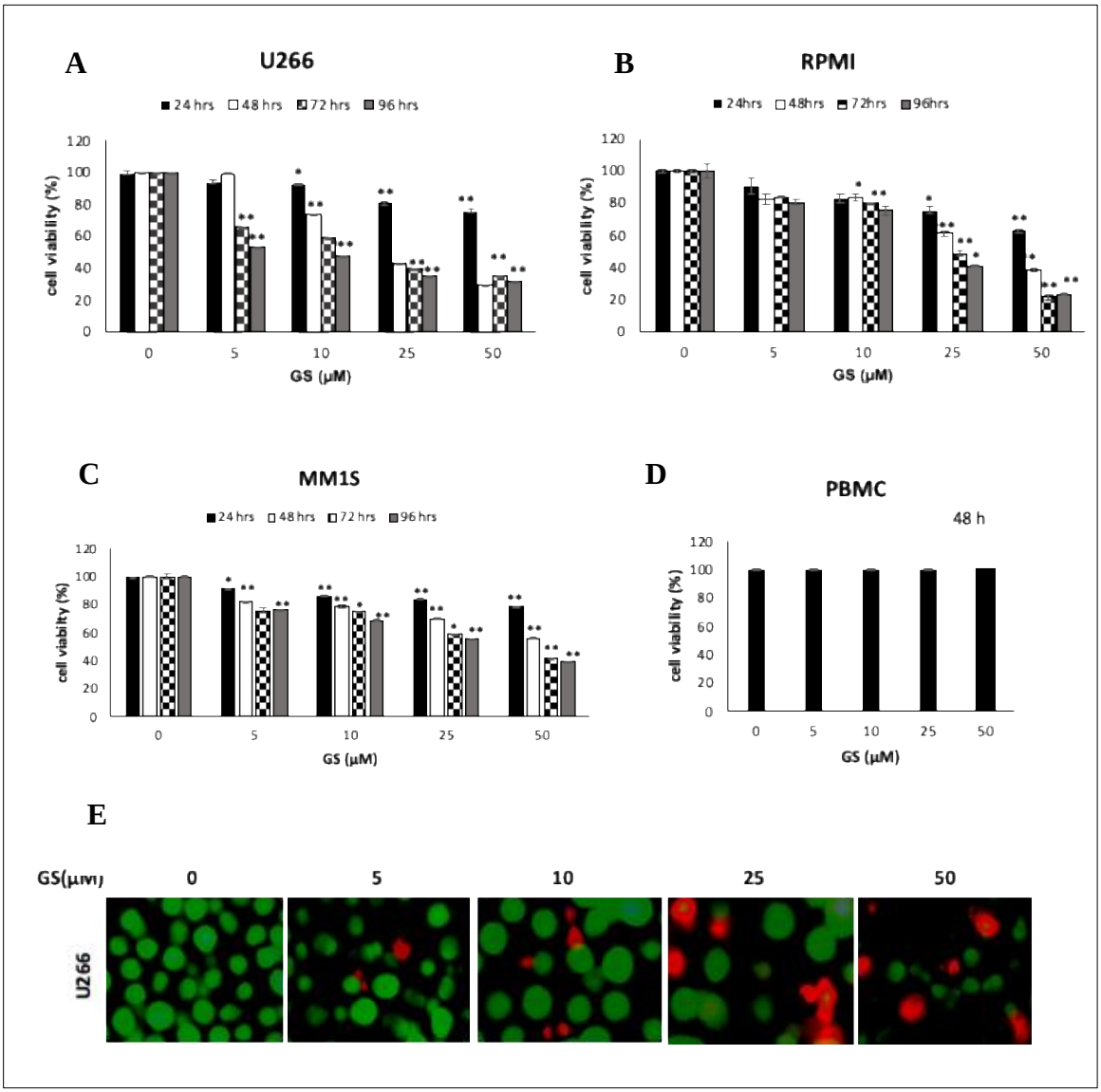

Figure 3: Effect of GS on MM cells. GS inhibits the viability of ***(A)*** *U266* ***(B)*** *RPMI* ***(C)*** *MM1S in a dose-dependent manner but does not inhibit the viability of* ***(D)*** *PBMC (healthy donor cells) after treatment with 0, 5, 10, 25, 50 µM of GS for 48 h. The graphs display the mean± SD (standard deviation) of three independent experiments with replicates. *p<0.05, **p<0.001.* ***(E)*** *Cell viability assessment performed using live/dead cytotoxicity kit at 0, 5, 10, 25 and 50µM GS treatment on U266 cells. Green indicates live cells, red indicator of non-viable cells.*

7.2. GS induces apoptosis and mediates cellular DNA damage in MM cells.

To confirm that the inhibition of MM cells viability occurs via the apoptosis cell death pathway, annexin V-FITC/PI dual staining experiment was performed using the flow cytometry technique.

The cell surface of healthy cells is composed of lipids distributed in the inner and outer plasma membrane. Phosphatidylserine (PS) is a lipid restricted in the inner plasma membrane and is exposed only to the cytoplasm. During apoptosis, PS is exposed to the outer plasma membrane, and it can bind to Annexin V, a calcium-binding protein. Furthermore, Annexin V can also stain necrotic cells due to their ruptured membranes. However, co-staining of Annexin V with propidium iodide (PI) can distinguish between necrotic and apoptotic cells as PI is membrane-impermeant in apoptotic cells but not in necrotic cells where the plasma membrane collapses (Crowley et al., 2016). Early stages of apoptosis when the cell membranes are still intact, stain positive for Annexin V-FITC but negative for PI (Annexin V-FITC^{+ve}/PI^{-ve}). However, late apoptotic cells are observed to be double-positive (Annexin V-FITC^{+ve}/PI^{+ve}), and in the necrotic stage, the plasma membrane integrity is lost, and cells stain negative for Annexin and positive for PI (Annexin V-FITC^{-ive}/PI^{+ive}).

Plots from figure 4 show non-apoptotic cell populations in the lower-left quadrant and early apoptotic cells in the lower right quadrant. Late apoptotic cells appeared in the upper right quadrant, and necrotic cells appeared in the upper left quadrant (Figure 4A).

Incubation of U266 cells with increased concentration of GS (0, 5, 10, 25, and 50 µM) resulted in a dose-dependent increase in apoptosis (5.4% ± 0.4 in control vs. 63.3% ± 1.1 with 50 µM GS) (Figure 4B). These findings are aligned with other studies where GS mediated apoptosis in other cancers such as breast, ovarian cancer, and

hepatocellular carcinoma (Shi et al., 2015); (Shishodia et al., 2007).

To get more insights into the mechanism of GS-mediated apoptosis in U266 cells, the activity of caspase -3/7 was examined using the caspase-3/7 green detection reagent and detected using an epifluorescence microscope. After 48 h of GS treatment, caspase 3/7 green detection reagent was added to cells as discussed in the methods section. This reagent is a nucleic acid binding dye with a DEVD peptide sequence that is a cleavage site for caspase-3/7. The dye is non-fluorescent until activation of caspase-3/7 in apoptotic cells where the DEVD peptide is cleaved, binding the dye to the DNA and producing a fluorescence response. U266 cells treated with increasing doses of GS, exhibited activation of caspase- 3/7 signals (Figure 4C).

Furthermore, double-stranded breaks (DSB) initiate genomic instability which ultimately lead to cancer. H2AX, from the histone H2A family is involved in the packaging of DNA into chromatin. In response to DSB, H2AX is rapidly phosphorylated (p-H2AX). Therefore, to assess the GS-mediated DNA damage in U266 cells, DNA double-strand breaks (DSB) were measured by quantifying p-H2AX (S139) using flow cytometry as described in the methods section. Bar graphs in Figure 5A indicate that the percentage of DNA breaks was equal to 4.3% ± 0.2 in untreated cells (control) and 11.9% ± 0.2 in cells treated with 50 µM GS. These findings were further supported by western blotting of the histone variant p-H2AX (Figure 5B). In fact, the formation of DSB triggers the activation of many factors, including phosphorylation H2AX. (Figure 5B).

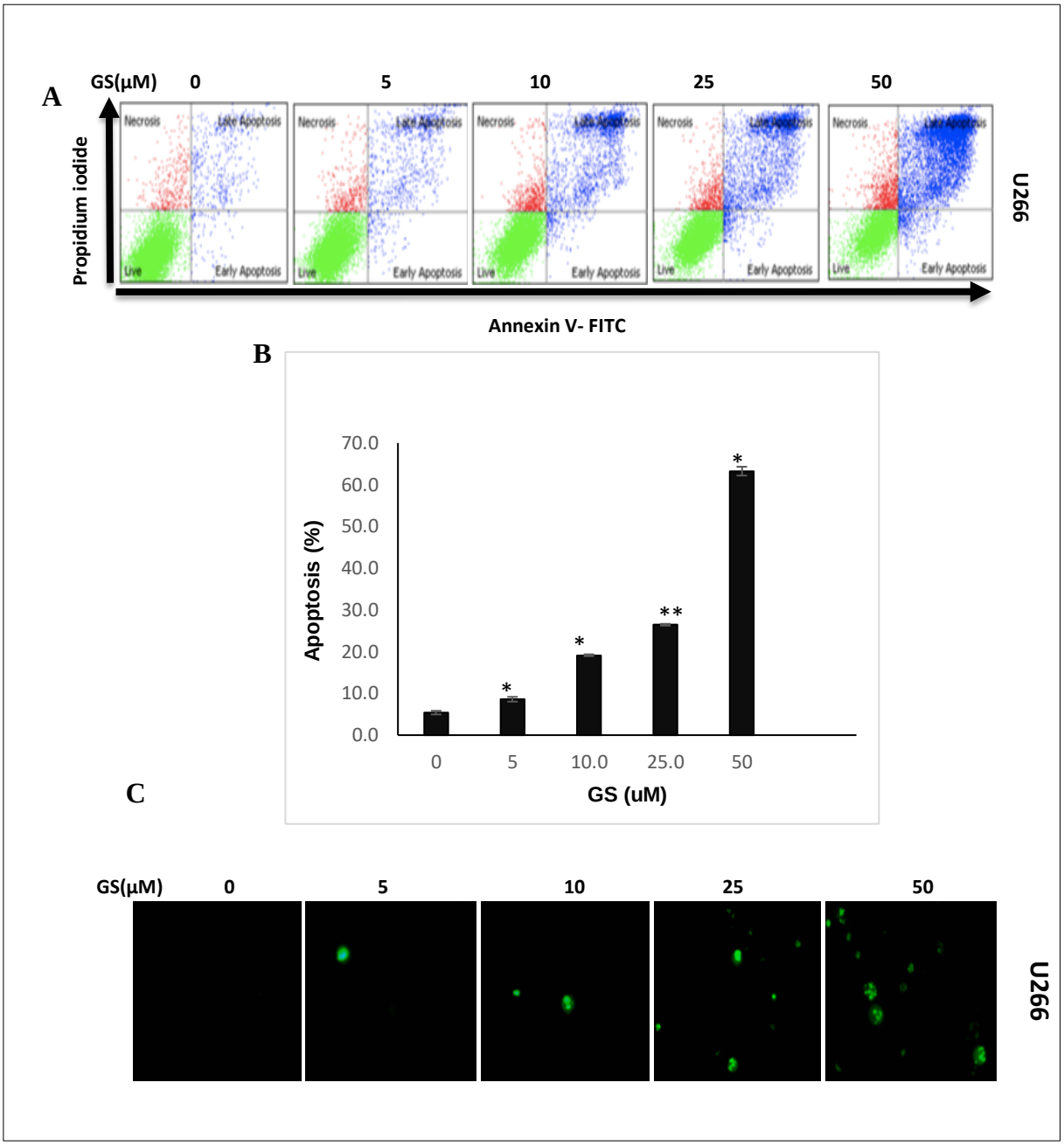

Figure 4: GS induced apoptosis in MM cells. ***(A)*** *U266 cells were treated with 0, 5, 10, 25, and 50 µM of GS for 48 h and stained with annexin-V-FITC, PI and analyszed using flow cytometry.* ***(B)*** *The graph displays the mean± standard deviation of the apoptotic cells percentage (Early and Late apoptosis) in three different experiments with replicates of three wells for all doses. *p<0.05, **p<0.001.* ***(C)*** *Apoptotic cells with activated caspase 3/7 depict the fluorescence green nuclei. U266 cells treated with different concentrations of GS for 48 h are shown.*

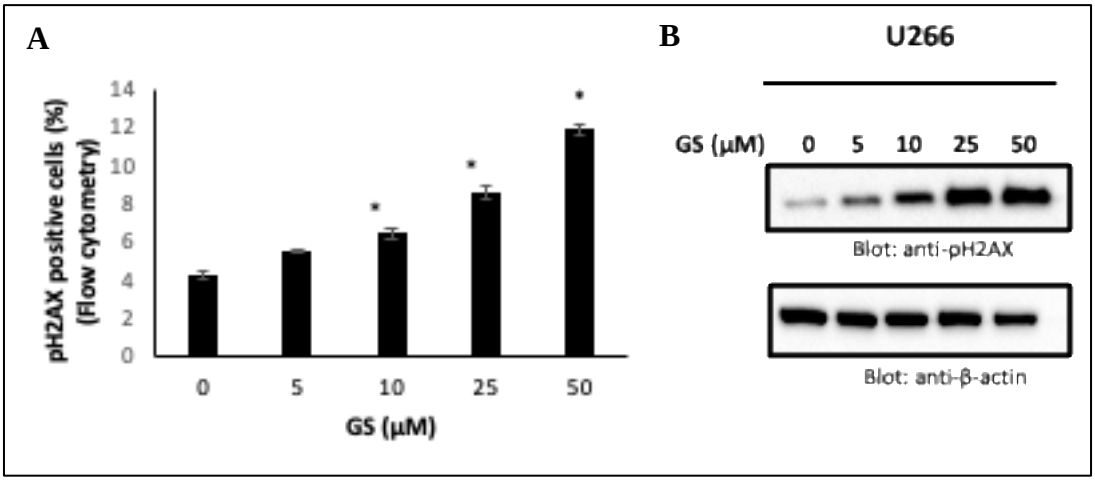

Figure 5: GS treatment prompts DNA damage in MM cells. ***(A)*** *U266 cells were treated with 0, 5, 10, 25, 50 µM of GS for 48 h, and quantified for DNA double stranded breaks using flow cytometry. The graph displays the mean ± SD of three independent experiments *p<0.05.* ***(B)*** *U266 cells were treated with increasing doses of GS for 48 h, lysed and immunoblotted against p-H2AX and β-actin for equal loading.*

7.3. GS activates the intrinsic apoptotic pathway in MM cells

The intrinsic apoptotic pathway is regulated by Bcl-2 family members primarily by affecting the mitochondria membrane homeostasis. Under stress conditions, proapoptotic proteins of the Bcl-2 family promote the permeabilization of the mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome-c in the cytoplasm. GS increased the expression of Bax and decreased the expression of Bcl-2 protein levels in U266 cells (Figure 6A). The expression of Bax and Bcl-2 proteins are involved in maintaining the mitochondrial membrane potential, whereby an intact mitochondrion prevents the leakage of cytochrome-c. Therefore, the next experiment was performed to measure the mitochondrial membrane potential (MMP). U266 cells were treated with GS (0-50 µM), labeled with JC-1 stain, and measured using flow cytometry. It was observed that MMP loss was 11.2% ± 1.9 in untreated U266 cells and increased significantly upon treatment with GS and reached a value of 50% ± 4.5 with 50 µM GS (Figure 6B).

The loss of MMP causes the release of cytochrome-c from the mitochondria. Therefore, in the next experiment, the release of cytochrome-c in U266 cells was investigated. U266 cells were treated with GS and immunoblotted with cytochrome-c and HSP60 for equal loading. As shown in Figures 6C and 6D, there is an increase in the expression of cytochrome-c following treatment with GS in a dose-dependent manner. On the other hand, the expression of cleaved caspase-8 was increased, whereas the expression of Bid protein was decreased, suggesting a truncation of Bid (and formation of t-Bid) (Figure 6E).

Apoptotic cell death is marked by the activation of caspases- 3, 6-, and 7-, known as executioner caspases. Western Blot experiments showed that treatment of U266 with GS resulted in the activation of caspase-3 and cleavage of PARP in a dose-dependent manner (Figure 7A). This result was further confirmed by determining the activation of caspase-3 and the subsequent cleavage of PARP using flow cytometry. U266 cells treated with GS resulted in a dose-dependent increase in the cellular levels of active caspase (3.1± 0.2 in control vs. 48% ± 0.1 in cells treated with GS 50 µM) and cleaved PARP (1.8% ± 0.3 in control vs. 59% ± 1.3 in cells treated with GS 50 µM) (Figure 7B and C). Moreover, GS-induced apoptosis activation of caspases was further investigated by the pretreatment of U266 cells with z-VAD-FMK, a universal inhibitor of caspases. Results showed that z-VAD-FMK prevented GS-mediated apoptosis and activation of caspase-3 and cleavage of PARP (Figure 7D). This confirms the involvement of caspase-3 in GS-mediated apoptosis. To validate this result, live/dead assay was performed. As expected, treatment of U266 cells with z-VAD-FMK alone and in combination with GS resulted in lesser dead cells as compared to cells treated with 50 µM of GS (Figure 7E).

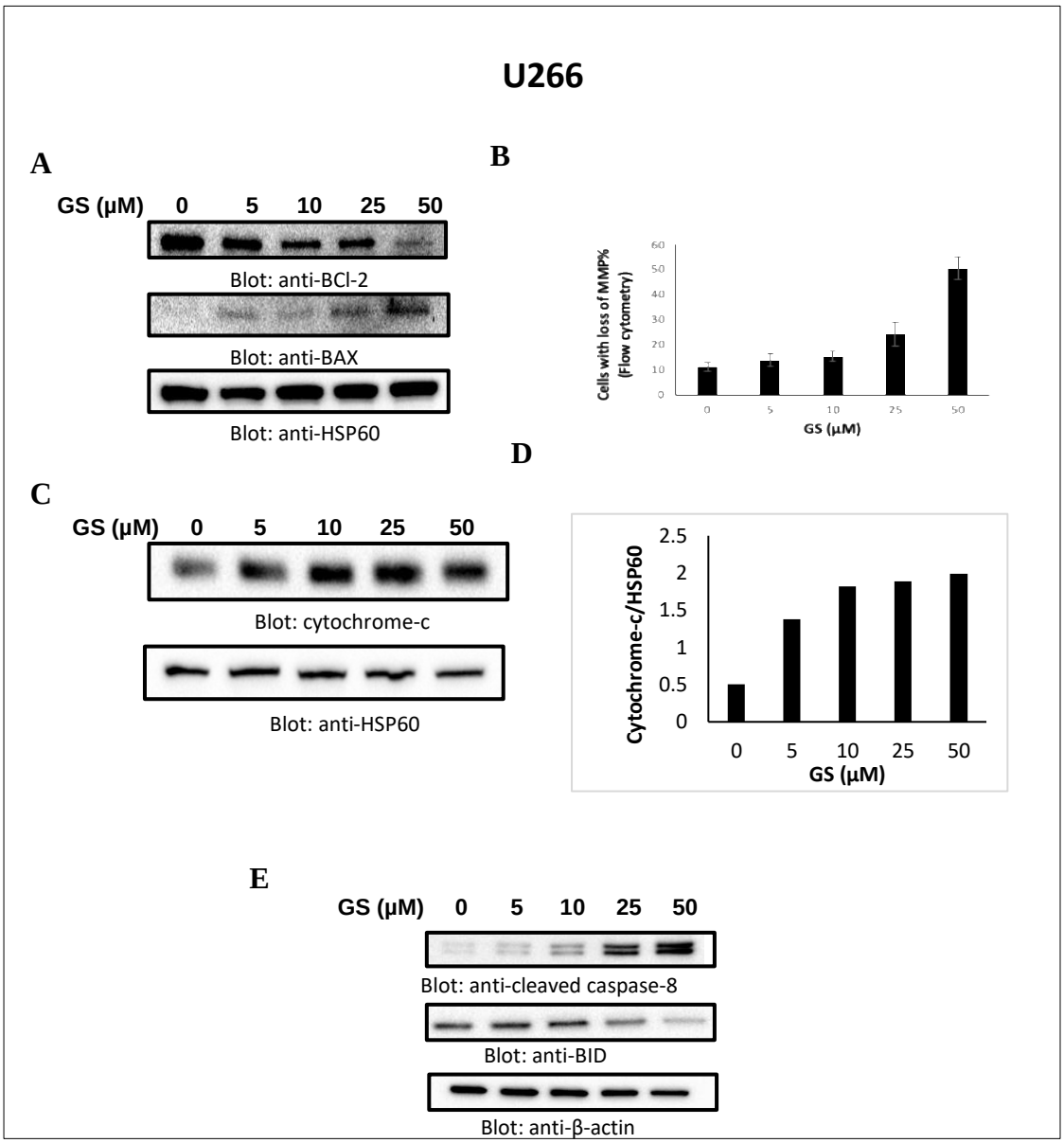

Figure 5: GS-mediated mitochondrial signaling pathways in MM cells. U266 cells were treated with increasing doses of GS for 48 h, cell lysates were prepared and immunoblotted with antibodies for ***(A)*** *Bcl-2, Bax, and HSP60.* ***(B)*** *GS treatment causes a loss of MMP in MM cells. U266 cells were treated with increasing doses of GS for 48 h. After JC1 staining, cells were analyzed by flow cytometry as described in Materials and Methods. The graph displays the mean ± SD of three independent experiments **p<0.001 ***p<0.0001.* ***(C)*** *Expression levels of cytochrome-c and HSP60 in U266 cells.* ***(E)*** *Densitometry analysis of cytochrome-c with HSP60.* ***(E)****Expression levels of cleaved caspase-8, Bid and β-actin.*

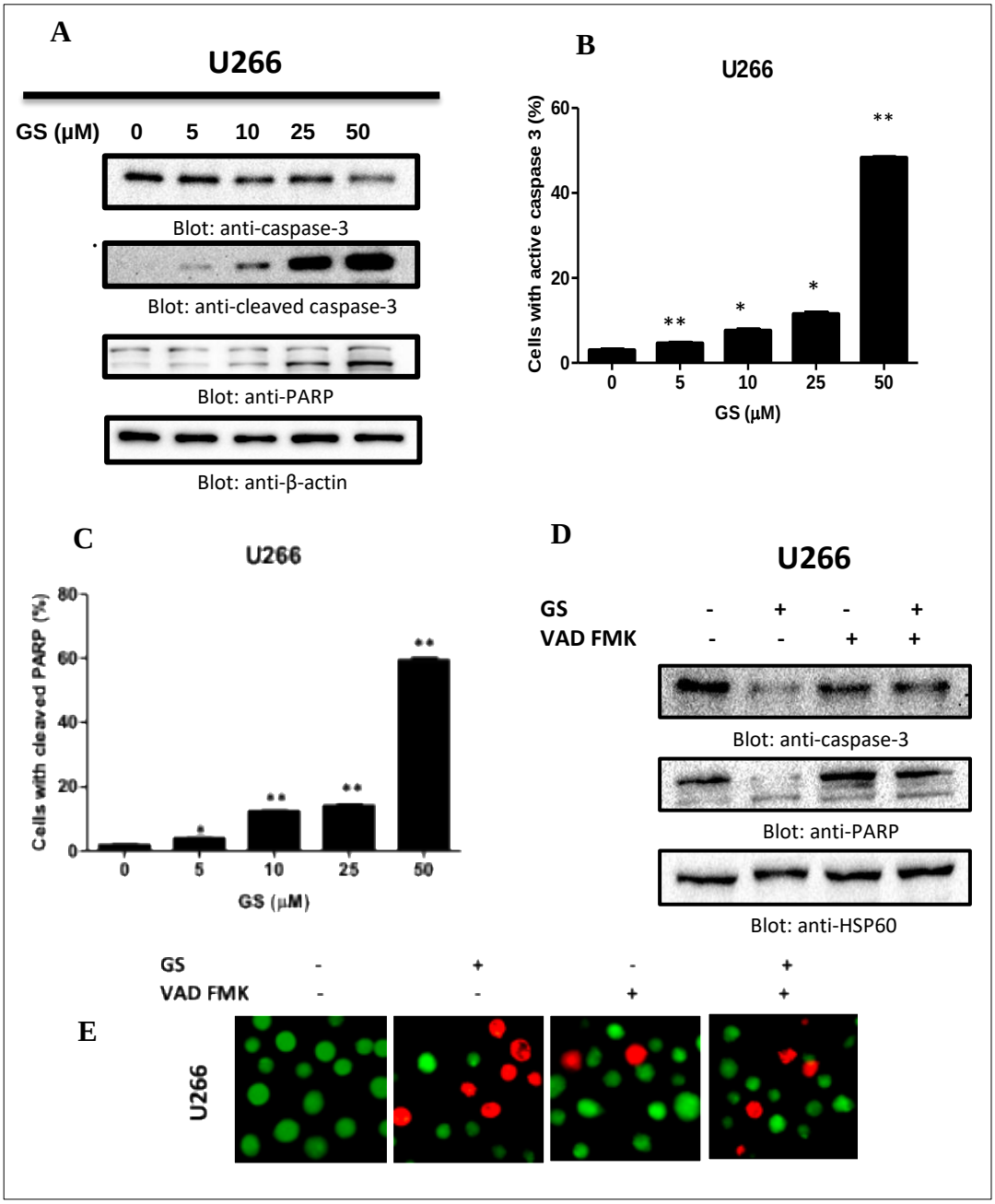

Figure 6: Activation of caspase cascade and PARP cleavage in MM cells are GS mediated. ***(A)*** *U266 cells were treated without and with 5, 10, 25, 50μM of GS for 48 h. After cell lysis, 50μg of proteins were separated on SDS-PAGE, transferred to PVDF membrane and immunoblotted against caspase-3, cleaved caspase 3, PARP, and β-actin.* ***(B)*** *GS- mediated activation of caspase-3 and* ***(C)*** *cleavage of PARP in U266 cell lines. The graphs display the mean ± SD of three independent of experiments *P<0.05, **P<0.01. U266 cells were pretreated with 30 μM z-VAD-FMK for 2 h, followed by 50 μM of GS for 48 h and cells were lysed and immunoblotted with* ***(D)*** *antibodies against caspase-9, caspase-3, PARP and HSP60 and stained using* ***(E)*** *live/dead kit.*

In the next set of experiments, we wanted to investigate whether the antiproliferative effect of GS on MM cells may involve other mechanisms of death than apoptosis, such as autophagy. Therefore, we assessed the expression of autophagy markers such as LC3, Atg5, and Atg7 proteins. Expression of LC3 is dependent upon the formation of autophagosomes, while Atg5 and Atg7 are considered essential proteins for the induction of autophagy (Gong et al., 2014). U266 cell lysates were separated on SDS-PAGE immunoblotted with antibodies against LC3, Atg7, Atg5, and GAPDH. It was found that LC3 is upregulated in U266 cells after GS treatment. Furthermore, Atg7 and Atg5 were observed to be downregulated (Figure 8). Similar findings were observed by (Cho et al., 2012).

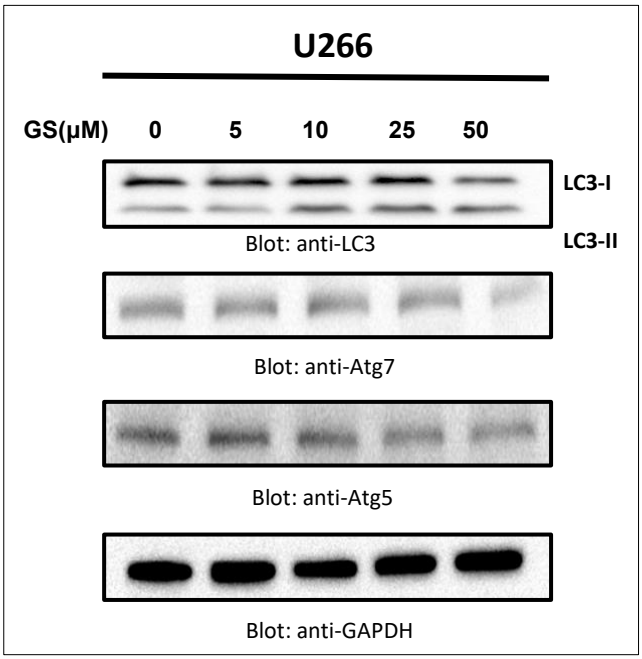

Figure 7: GS induces autophagy in MM cells. U266 cells were treated with GS in a dose-dependent manner for 48 h. The cells were lysed, separated by SDS-PAGE, and immunoblotted with antibodies against LC3 II, Atg7, Atg5 and GAPDH for equal loading.

7.4. GS treatment downregulates the expression of HMGB-1 via JAK/STAT pathway

To assess the role of HMGB-1 in U266 cells treated by GS, HMGB-1 ELISA was performed. The results indicated a significant decrease in the expression of HMGB-1 in U266 cells after 48 h of GS treatment 2000pg/ml ± 0.3 of HMGB-1 in control vs. 1000pg/ml ± 0.8 in cells treated with 50 µM of GS (Figure 9A). In addition, western blot analysis confirmed the downregulation of HMGB-1 in MM cells after treatment with GS (Figures 9B and 9C).

Furthermore, to understand the role of HMGB-1 within JAK/SAT pathway in MM cells, U266 cells were treated with GS and AG-490, a JAK2 inhibitor for 48 h, and protein lysates were immunoblotted with antibodies against p-STAT3, STAT3, and HMGB-1. The results demonstrated that treatment of U266 with GS alone and in the presence of AG-490 showed a decrease in the expression of p-STAT3 and HMGB-1 (Figure 9D). Previous studies have shown that suppression of STAT3 using specific inhibitors suppressed the growth of cancer cells (Zhang et al., 2015). AG-490 is reported to significantly decrease the expression of p-STAT3 at a dose of 50 µM (Zhang et al., 2015). However, U266 cells treated with AG-490 did not depict a decrease in the p-STAT3 levels. This can be explained by the use of a tiny amount of AG-490, (i.e., 20 µM). On the other hand, for the same concentration, HMGB-1 expression is decreased, which indicates that HMGB-1 expression is dependent on the activation of JAK/STAT signaling pathway. In addition, the combination of GS and AG-490 exhibits a downregulation of p-STAT3 and HMGB-1, therefore, depicting the involvement of HMGB-1 and JAK/STAT signaling pathway in MM pathogenesis. Also, a decrease in the expression of HMGB-1 can be an indicator of suppression of MM growth. No changes were observed in total STAT3 after treatment with AG-490 and GS, which is consistent with previous studies (Gurbuz et al., 2014); (Ahn et al., 2008). Moreover, to

further validate the downregulation of HMGB-1 via JAK/STAT pathway, a knockdown of STAT3 using siRNA was performed. As shown in Figure 9E, STAT3 specific siRNA decreased STAT3 protein levels, which downregulated the expression of HMGB-1(Figure 9F and 9G).

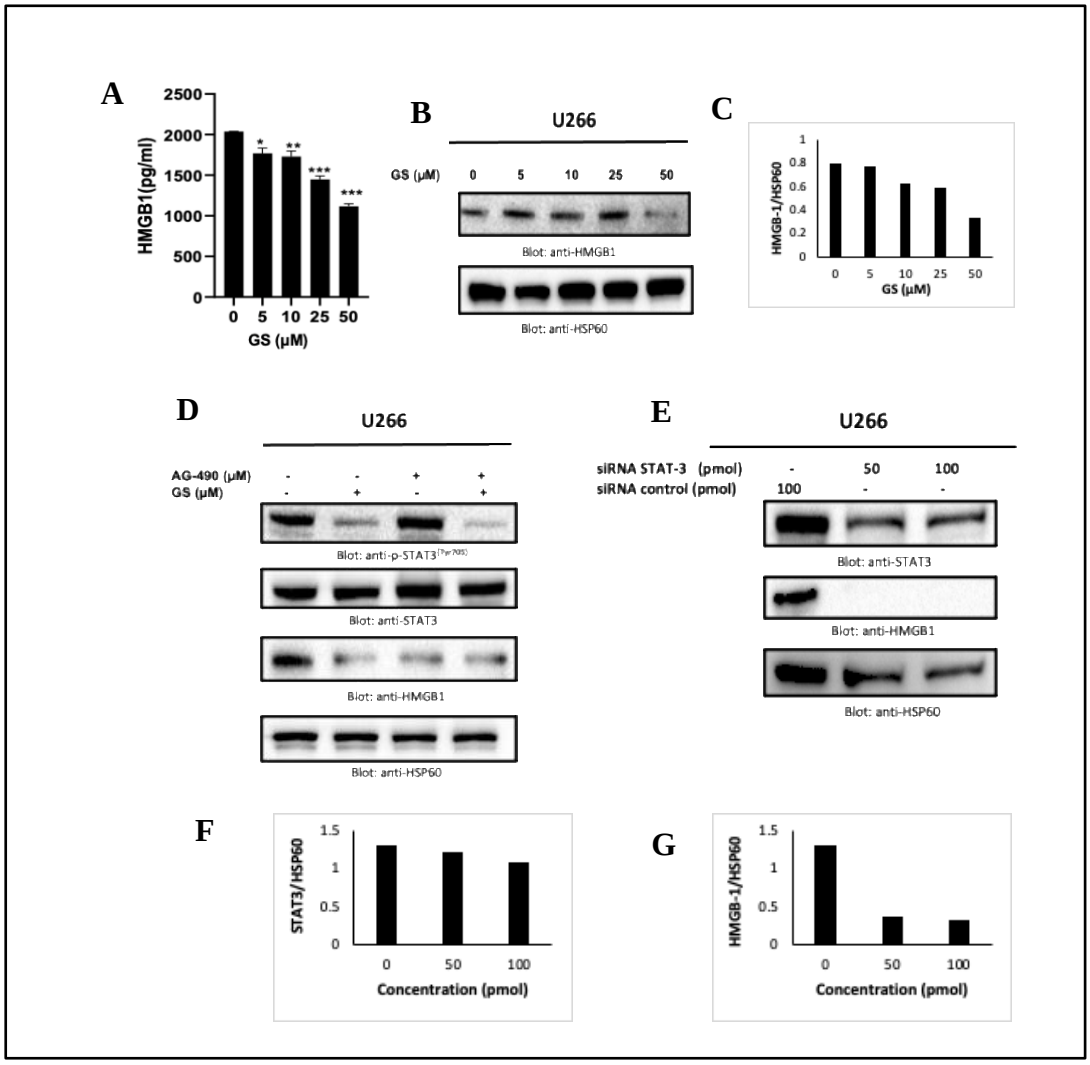

Figure 8: GS downregulates HMGB-1 expression via JAK/STAT signaling pathway. ***(A)*** *U266 cells were treated with GS for 48 h. The cell supernatant is collected and measured for levels of HMGB-1 using ELISA. The graph is expressed in mean±SD of three independent experiment. *p<0.05, **p<0.001, ***p<0.0001.* ***(B)*** *U266 cells were treated without and with 5, 10, 25, 50μM of GS for 48 h. After cell lysis, 50μg of proteins were separated on SDS-PAGE, transferred to PVDF membrane and immunoblotted against HMGB-1 and HSP60.* ***(C)*** *Densitomery analysis of HMGB-1/HSP60 blots* ***(D)*** *U266 cells were treated with 10 μM GS and 20 μM of AG-490 for 48 h. After cell lysis, 50 μg of proteins were separated on SDS-PAGE, transferred to PVDF membrane and immunoblotted against p-STAT3, STAT3, and HMGB-1.* ***(E)*** *Effect of STAT3 knockdown on total STAT3 and HMGB-1 expression. Densitometry analysis of* ***(F)*** *STAT3* ***(G)*** *HMGB-1 with HSP60.*

7.5. GS constitutively suppresses activated STAT3 signaling pathway and inhibits IL-6 induced STAT3 activation in MM cells

Many studies have identified STAT3 as a crucial mediator of cell survival during cancer development. The malignant behavior of MM cells is primarily driven by the activation of STAT3 and its downstream targets. Therefore, to understand the oncogenic role of STAT3 and the effect of GS on this signaling pathway, U266 cells were treated with increasing doses of GS and immunoblotted with antibodies against p-STAT3, STAT3, Bcl-xl, XIAP, and cyclin D1. It was found that GS treatment inhibited the phosphorylation of STAT3 at Tyr705, in a dose-dependent fashion (Figure 10A). However, there was no effect of GS on total STAT3 protein levels (Figure 10A). In addition, GS treatment of U266 cells resulted in a decrease in the expression of STAT3 downstream targets such as Bcl-xl, XIAP, and cyclin-D1 (Figure 10A).

Furthermore, Protein Tyrosine Phosphatases (PTPs) are negative regulators of the JAK/STAT signaling pathway (Valentino & Pierre, 2006). Therefore, in the following experiments, we investigated the involvement of PTPs such as SHP-1 in GS-mediated phosphorylation of STAT3. Figure 10B shows that GS treatment resulted in the upregulation of SHP-1 expression in a dose-dependent manner. Similar observations were made by (Ahn et al., 2008), where SHP-1 was found to be upregulated upon GS treatment.

STAT3 has been shown to be activated by various cytokines, specifically IL-6. Therefore, in the next part of the study, the effect of GS on IL-6 secretion and IL-6 mediated STAT3 activation was investigated. U266 cells were treated with GS for 48 h, and the secretion of IL-6 in the media was measured using a multiplexing kit. We found that the secretion of IL-6 was significantly reduced after GS treatment, markedly with 25 and 50 µM GS. Untreated cells showed a basal release of 0.95 pg/ml ± 0.03 of

IL-6, while in the presence of 25 and 50 µM GS, IL-6 concentration was found to be 0.61 ± 0.06 and 0.56 ± 0.01 pg/ml, respectively (Figure 11A). In addition, to investigate IL-6 mediated STAT3 activation, U266 cells were serum-starved for 24 h, and then treated with 25 µM GS for 1 h and then stimulated with IL-6 (100µg/ml) for 30 min. After preparations of cell extracts, immunoblotting was conducted using antibodies against p-STAT3 and STAT3. Incubation of U266 cells with IL-6 has been shown to increase the phosphorylation of STAT3 (Figure 11B). However, incubation of U266 cells with GS alone and with GS and IL-6 prevented IL-6-mediated STAT3 phosphorylation, suggesting that GS inhibits the IL-6-induced activation of STAT3.

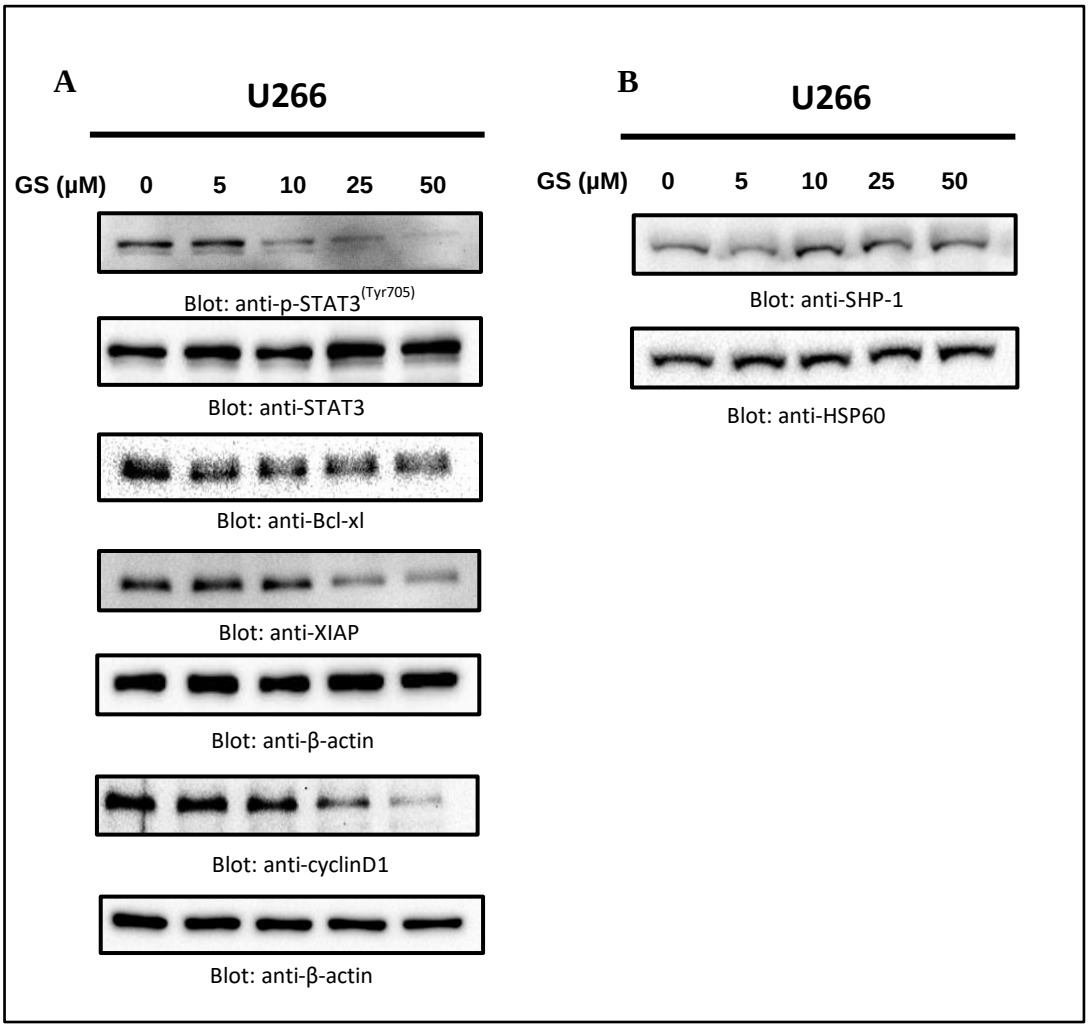

Figure 9: GS suppresses activated STAT3 signaling pathway. ***(A)*** *U266 cells were treated with increasing doses of GS for 48 h. After cell lysis, equal amounts of proteins were separated by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with antibodies of p-STAT3 (Try705), STAT3, Cyclin D1, Bclxl, XIAP, and GAPDH.* ***(B)*** *Involvement of PTPs in GS induced deregulation of STAT3. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with antibodies of SHP-1 and HSP60.*

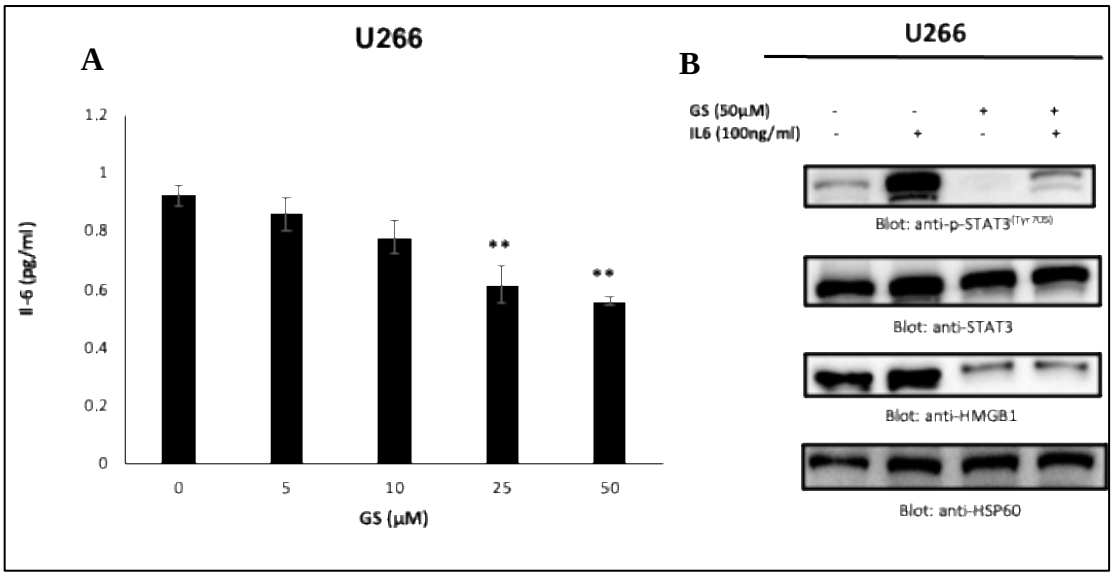

Figure 10: GS mediated inhibition of IL-6 secretion and IL-6 induced STAT3 activation in MM cells. ***(A)*** *GS treated U266 cells supernatant was collected and a quantitative analysis for IL-6 was performed using multiplexing kit. The graph displays mean±SD of three independent experiments. **p<0.001.* ***(B)*** *U266 cells were serum starved for 24 h, subsequently pretreated with 50µM GS for 48 h and then stimulated with IL-6 (100 ng/ml) for 30 min. Cell extracts were prepared, immunoblotting performed using antibody against p-STAT3, STAT3, HMGB-1 and HSP60.*

8. DISCUSSION

MM is one of the fastest progressing cancers globally. There are several chemotherapeutic drugs and therapies targeting the suppression of MM cells. However, MM cells often become resistant to these treatments, which urges the need for novel anticancer agents. Guggulsterone, a phytosteroid of the plant *Commiphora mukul,* has been reported to possess anti-inflammatory and antioxidant properties. Hence, in this study, we sought to evaluate the anticancer potential of GS on MM cells. Data obtained from this study showed that GS inhibited the proliferation of MM cells in a dose-dependent manner. Moreover, GS induces MM cell death via the intrinsic apoptotic pathway that involves caspases activation and cleavage of PARP. Potentially, crosstalk with the extrinsic pathway via an amplification loop that involves the activation of caspase-8 has been depicted.

It is well established that caspases are drivers of apoptotic cell death and cleave other cellular proteins such as PARP that are critical players involved in the dismantling of the cell (Loreto et al., 2014)). Moreover, PARP is reported to increase 100-folds in case of DNA damage and contributes to cell death (Zhang et al., 2012). Also, loss of MMP and subsequent release of cytochrome-c has been reported in various studies (Prabhu et al., 2017); (Kantari & Walczak, 2011). Furthermore, the release of cytochrome-c stimulates the formation of apoptosome, a complex composed of Apaf-1, caspase 9, and cysteine protease. Active caspase-9 further activates the effector caspase-3 and induces cell death. In this study, the expression of active caspase-3 and caspase-9 were assessed (data not shown for caspase-9). In addition, caspase-3 may also activate caspase-8, the downstream effector of the extrinsic pathway of apoptosis. Caspase-8 will then cleave Bid into t-Bid that undergoes conformational changes and amplify the intrinsic apoptotic pathway by releasing cytochrome-c. Our results are

consistent with previous studies (Basu et al., 2006); (McComb et al., 2019). Therefore, we suggest the activation of the intrinsic apoptotic and extrinsic pathways in MM cells when treated with GS. Moreover, GS caspase-dependent apoptotic effect was investigated using z-VAD-FMK, an inhibitor of caspases, which prevented GS-mediated apoptosis. This further confirmed the involvement of caspases in GS-induced apoptosis. However, since there was a partial reversal of cell survival, we inferred the existence of other mechanisms of cell death induced by GS.

We next sought to determine the involvement of another GS-mediated death mechanism, such as autophagy. Autophagy prevents the accumulation of damaged organelles and proteins in tumor cells. In the cytosol, LC3-I conjugates with phosphatidylethanolamine and forms LC3- phosphatidylethanolamine conjugate (LC3-II), which is then recruited to the autophagosomal membrane. The fusion of autophagosomes with lysosomes leads to the formation of autolysosomes that are degraded by lysosome hydrolases. Consequently, LC3-II in the autolysosomal lumen is also degraded. Thus, LC3-II is one of the most prominent autophagy induction markers (Tanida et al., 2008). In our study, after GS treatment of U266 cells, an increased expression of LC3 protein indicated autophagy induction.

Moreover, autophagy-related proteins such as Atg5 and Atg7 are also associated with various cancers (Kang et al., 2009). GS treatment decreased the expression of Atg5 and Atg7 in MM cells. This suggests the suppressive role of these autophagy proteins in tumorigenesis. In the early stages of tumorigenesis, autophagy prevents chromosomal stability, organelle normality, and genomic integrity, therefore, suppressing the tumor (Yang et al., 2011). Also, studies have reported the induction of autophagy as a mechanism that could enhance sensitivity to radiation and chemotherapy in malignant cells (Li et al., 2013).

As mentioned previously, HMGB-1 is actively involved in various processes of cancer such as tumor growth, tumor cell proliferation, metastasis, and invasion (Tang et al., 2010). **This makes HMGB-1 a critical target in cancer therapy. In this study, for the first time, the role of HMGB-1 in MM cells was elucidated, and it was identified that GS mediates the downregulation of HMGB-1 in MM cells.** In addition, the role of HMGB-1 in JAK/STAT signaling pathway was studied. As highlighted in recent studies, deregulation of JAK/STAT pathway causes resistance to apoptosis in multiple human malignancies (Thomas et al., 2015); (Akhtar et al., 2019). Inhibition of JAK-2 using specific inhibitors such as AG-490 resulted in the downregulation of HMGB-1 protein levels. Moreover, upon siRNA knockdown of STAT-3, there was a remarkable decrease in the expression of HMGB-1.

Interestingly, this depicted that HMGB-1 expression is dependent on the activation of JAK/STAT pathway. In addition, activated STAT3 controls the transcription of various antiapoptotic genes such as Bcl-xl. Upregulation of Bcl-xl suppresses apoptosis and enhances the resistance to chemotherapy. In our data, GS suppressed the phosphorylation of STAT3, thereby downregulation the expression of STAT3 downstream targets, including Bcl-xl, cyclin D, and XIAP. In addition, protein tyrosine phosphatases such as SHP-1 regulated the activation of STAT3. Our findings depicted that GS treatment upregulated the expression of SHP-1 and is associated with the decrease in phosphorylation of STAT3 in U266 cells.

The prevention of IL-6 mediated STAT3 activation due to other anticancer agents in MM cells has been described before (Li et al., 2010); (Akhtar et al., 2019). Our results align with these studies. GS also inhibited IL-6 secretion and IL-6 mediated activation of STAT3 in U266 cells. Interestingly, treatment of GS with IL-6 resulted in suppression of p-STAT3 and downregulation of HMGB-1. Therefore, we conclude that

GS inhibits IL-6 secretion that suppresses the activation of STAT3, thereby downregulating the expression of HMGB-1 and inhibiting MM cell proliferation.

In conclusion, this study underscores the role of GS as an anticancer agent and depicts the underlying molecular mechanisms involved in the pathogenesis of MM. Importantly, we described the GS-mediated downregulation of HMGB-1 and suppression of STAT3 activation in MM cells. Given the emergent role of HMGB-1 in MM pathogenesis and as potential target for cancer therapy, it will be worth studying the multifaceted role of HMGB-1 in cancer development and progression. More specifically, the role of HMGB-1 in MM cell growth, migration, and tissue invasion needs more investigations. Finally, in vivo studies and clinical assays are required to explore the therapeutic efficacy of GS in MM and other types of cancer.

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